

Britain's very own Brundtland strategy

Good intentions on sustainable development have plainly captivated the British government's heart, but its now published strategy should not be mistaken as a basis for public policy.

SUSTAINABLE development may be politically correct, but is it politically accepted, or even acceptable? That has been the position for the past several years, since the publication in 1987 of *Our Common Future*, the report of an international commission under Mrs Gro Harlem Brundtland, the prime minister of Norway. One consequence was the decision of the United Nations that there should be a UN Conference on Environment and Development at Rio de Janeiro two years ago, which in turn led to international treaties on climate change and biodiversity, to a specific plan for the conservation of forests and a loosely argued wish-list of actions for the world at large (called "Agenda 21") that would, if followed, make sustainable development possible.

The British government seems to be persuaded that there will be no great difficulty. Last week it published a sheaf of documents* (on recycled paper, with even the varnish on the covers said to be fully recyclable) which together provide a comprehensive account of where British environmental policy now stands, and which promise the government's firm commitment to sustainable development. Now, putting the horse behind the cart, the government has arranged a debate on what it proposes in the House of Commons next Monday (7 February). It will be interesting to learn whether politicians know what Mr John Major, the prime minister, let them (and their constituents) in for when he flew off to Rio in 1992.

Improvement

First, the good news. Britain has a decent record on environmental protection, a much better one than its domestic critics allow. Last week's publications amply document that. Air and water quality have improved greatly over the past couple of decades. Forests are growing in acreage and even in diversity. Industrial activities such as minerals extraction (mostly, now, in Britain for building aggregates) and chemical sources of persistent pollution are well regulated and controlled. Whatever may be the grumbles of the government's supporters at the regulations that flow from the European Union in Brussels, Britain dutifully implements them even in the face of contrary advice (as with the declaration of its own Standing Commission on Environmental Pollution that nitrate standards for drinking water

need not be so stringent).

The bad news is that the British government, perhaps reflecting the island character of its domain, has chosen to look through the wrong end of the telescope. It is plainly hoping to justify its support for sustainable development by an impressive parade of detail, when the truth is that sustainable development is nothing if not a global problem. That is what the Brundtland Commission implied when it defined its cause as "development that meets the needs of the present without compromising the ability of future generations to meet their needs". Readers of the government's new tomes will not easily be able to see how these lofty goals are likely to be furthered by the admittedly fascinating plan to reintroduce the large blue butterfly at half a dozen sites in Britain, which entails keeping the sites closely grazed and supplied with wild thyme and red ants, essential props of the blue butterfly's life-cycle. God may be in the detail, but the Devil (in the shape of the costs of sustainable development) struts on a wider stage.

Comfort

The comfort is that the British government is not alone in its error, for which there are several causes. One is insularity (often also encountered in landlocked places). That consists of the supposition that if each nation-state ensures sustainability in its own domain, everything everywhere will be all right. The trouble is that where global problems are concerned, national efforts cannot ensure national sustainability. If Britain acts prudently on carbon dioxide emissions, that will not save British skins if, say, China does not care or Russia cannot. Worse, even less extensive problems are best dealt with internationally. If the purpose of growing forests, for example, is as a sink for carbon dioxide, it is best to put them where they grow fastest and can be harvested with the least amount of trouble. Interdependence is a fact of modern life only reinforced by environmental problems. Britain's government seemed to have overlooked that.

Another error is the supposition that environmental goods are priceless or, rather, free from cost. Although last week's sheaf of documents refers repeatedly to the inconvenience that environmental policies may cause, it makes no attempt to estimate what the costs will be, let alone the benefits. Everybody acknowledges that this is a tricky area, but that does not excuse the almost complete neglect in these documents of the economics of the policies outlined. Yet the truth

* *Sustainable Development: The UK Strategy; Sustainable Forestry: The UK Programme; Biodiversity: The UK Action Plan; Climate Change: The UK Programme* (HMSO, 1994).



is no different now from what it has always been. Environmental benefits are public purchases made ultimately by a country's voters (as taxpayers or consumers). It is worse than an oversight that this ragbag of policies — some are imaginative — should have been lumped together under the name of "Strategy" without even an attempt to put them in qualitative order of priority. To be sure, there are soothing phrases here and there. The documents say that the government will ensure that "environmental costs and benefits are properly and fully taken into account. So do global warming and the blue butterfly have equal weight?

There is finally the error of false perspective. Over what period does Britain plan for sustainable development? The documents last week say 20 years, although at one stage the document on biodiversity looks forward to the emergence of new animal species in Britain, from which glacial ice has vanished for only 10,000 years or so. But in reality, even 20 years is too long for safe planning ahead. On a mundane level, Britain's membership of the European Union is unlikely to leave net immigration rates at 0.1 per cent (especially if the cost of sustainability requires further tax increases).

More generally and more important, the calculations suppose there will be no noticeable improvement of people's cleverness, which is amply denied by even recent experience. Those who doubt that have only to consider what sustainable policy the British government would have devised for its coal industry if sustainable development had been fashionable in the 1920s, when output was 250 million tonnes a year. (Ironically, one of the government's chores in the months ahead is to sell the remnants of that industry to whoever will buy them.) In the event, it proved possible to drill for oil in the North Sea instead of mining coal, of which there is plenty still beneath Britain's surface. The best hope that "future generations will be able to meet their needs" remains what it has always been; that future generations will be smarter than their predecessors.

So is the policy now on offer acceptable? The British government will win the approval of the House of Commons after three hours of probably desultory debate — British MPs have other things on their minds — are will reckon that its democratic duty is done. The new documents promise a process of continuing consultation, and of public education, which makes sense. But the years ahead should also be spent in filling in the obvious gaps in a strategy that is too shapeless to be a guide to action and, despite the entrancing detail, too vague to be refined. □

Hearing for basic science

US leaders of all disciplines have met to ponder the importance of fundamental science.

A YEAR ago, US President Bill Clinton offered science and technology a central role in the future of the United States in a White House paper called *A Vision of Change*. Vice-President Al Gore, chief advocate of the electronic

superhighway for data exchange, had clearly had a hand in the document, which spoke glowingly of programmes to enhance US competitiveness in international markets and made clear his support for "strategic research" in such areas as advanced materials and improved manufacturing techniques. It was a dream come true for those in the United States who want a clear policy of government support for new technology, but not everybody was delighted. For many researchers, the president's vision carried another message: that the president does not really understand basic science, research without targets.

In an effort to change at least that perception, the White House Office of Science and Technology Policy (OSTP) has planned for this week a major meeting, called a convocation, as a prelude to a presidential proclamation of the virtues of basic science. Participants — all by special invitation — will by now have spoken on the role of basic research in a variety of contexts — biology, industrial work, national security and economics among them. Scientists, educators and members of the US House of Representatives and Senate whose committees have jurisdiction over science agencies were on the list. So too was John Deutch from the Department of Defense. Senator Jay Rockefeller (Democrat, West Virginia) was on the programme, as were Harold Varmus, director of the National Institutes of Health (NIH) and Neal Lane, head of the National Science Foundation (NSF). Even Vice-President Gore has agreed to attend, perhaps to show that he is not really an unreconstructable technocrat.

What will the meeting have accomplished? It would be naive to think that a single presidential policy statement will reverse the declining budgets of agencies such as NSF or NIH, whose research support is anyway moving slowly if perceptibly towards targeted science. In any case, the exact magnitude of the agency budgets is less important than the long-term benefits to US society of economy of the products of basic research, which are two: understanding and skilled people. From the design of silicon chips (and the software to go with them) to gene therapy and its infrastructure, the administration should by now be clearly aware that US prowess in these and other fields rests squarely on the former and has been won only by the latter. Yet there are already fields in which US industry is cramped for lack of sufficient skill, of the kind that can be acquired only through training by research. The lesson, which should need no telling, is that to skimp on basic research will undermine the goals on which the administration set its heart just a year ago.

In that sense, it is a pity that this week's high-powered gathering has been necessary at all. But the plot may be thicker than it seems. The convocation is the brainchild of M. R. C. Greenwood, associate director for science in the White House, who in a previous life was dean of graduate studies at the University of California at Davis. Greenwood is not only committed to federal support of basic science but also shrewd enough to know that a white paper from the White House may be politically valuable in future budget battles. Indirectly, the great and the good may have been giving evidence to the Congress. □

Clinton administration blows cool on US involvement in Europe's LHC

Washington. Hopes that the United States will help to fund the construction of the Large Hadron Collider (LHC) at the European Laboratory for Particle Physics (CERN) in Geneva received a jolt last week when President Bill Clinton's science adviser, John Gibbons, failed to back the idea in a presentation to Congress.

High-energy physicists have been hoping that, following the cancellation of the Superconducting Super Collider (SSC) in Texas last October, the way had been opened up for greater US involvement in the LHC. They were therefore shocked when Gibbons failed to mention CERN by name in his testimony to a hearing of the House science subcommittee chaired by Rick Boucher (Democrat, Virginia).

According to congressional staff, the hearing had been intended to explore options for US participation in the LHC. But Gibbons instead appeared to suggest that the United States should lead a multinational effort to build an even more powerful accelerator at some future date. "Eventually a new facility will be needed," he said, after promising to maximize the use of existing US accelerators. "It should be a multinational effort, and the US should adopt a leadership role."

Asked by Sherwood Boehlert (Republican, New York) about the "conspicuous absence of any reference" to CERN in his testimony, Gibbons said it was of "no significance". But he added that attaching the future of US particle physics to CERN would be "to tie it solely with Europe, which would not be good for either party at this stage".

Despite Gibbons' denials, congressional staff are concerned that his reluctance to make any commitment to CERN will make it impossible to strike a deal with the laboratory's European members, and persuade a deeply sceptical Congress to accept it.

They fear that Gibbons may be softening up the high-energy community for budget cuts — not just in next week's budget for the 1995 financial year, but in succeeding years as well — as the importance of the field is reassessed in the wake of the SSC's demise.

Physicists told Boucher's subcommittee that formal US participation in the LHC was vital for the future of US high-energy physics, and would cost between \$500 million and \$1 billion over ten years.

John Peoples, director of the Fermi Laboratory, said he estimated that US participation in CERN would cost around \$300 million for the construction of the LHC, and \$200 million for detector work. Frank Merritt of the University of Chicago said the cost of supporting physicists working at CERN

would be between \$50 and \$60 million per year, while an "appropriate level of [US] support" for CERN's overall costs would be between \$30 and \$40 million.

A subgroup of the Department of Energy's High Energy Physics Advisory Panel (HEPAP) will report in the spring on what priority such an investment should have in the department's \$620 million budget for high-energy physics.

Boehlert, one of the leaders of the congressional campaign against the SSC, is one of several subcommittee members now keen to support US participation in CERN. But the position of Joe Barton (Republican, Texas) may be more representative of Congress as a whole.

"This congressman is going to look very sceptically — very sceptically — at us trotting over to Europe, given that they have done absolutely nothing to help with what was the biggest high-energy physics project in the world," said Barton. "I never saw one mark, frank or pound coming to this side of the ocean [for the SSC]."

Administration officials sympathetic to CERN say that such views can be overcome only if top officials such as Gibbons and Hazel O'Leary, the Secretary of Energy, strongly support the collaboration and sell it hard to Congress.

Martha Krebs, director of energy research

at the Department of Energy, held her first meeting with Chris Llewellyn-Smith, the director of CERN, last Saturday, 29 January, when both were in California. Krebs says that "internal discussions" in her department have looked at various options for involvement in CERN.

These options include full membership, associate membership or some form of bilateral partnership. But Krebs says that no official discussions have yet taken place. "It's something we are just beginning to pull together," she says.

There will clearly be no money for CERN participation in the budget for fiscal year 1995, which begins on 1 October. Gibbons says consideration will be given to including such money in the budget for fiscal year 1996, once the HEPAP panel has delivered its report, and the president's new National Science and Technology Council (NSTC) has considered the matter.

But it seems unlikely that the NSTC will want to increase the United States' annual expenditure on high-energy physics. And even if HEPAP makes collaboration with CERN a higher priority than existing, US-based facilities such as Fermilab, it is hard to see what the Energy Department, the administration or Congress would have to gain from transferring money from these US facilities to Europe.

Colin MacIlwain

Academy hotel deal reaps hidden rewards

Moscow. Suspicions that the Russian Academy of Sciences may not have been entirely open about the details of the recent sale of property in the centre of Moscow have been heightened by the discovery of a safe containing almost US\$1 million in one of the academy's former buildings.

Several years ago, the praesidium of the academy reached agreement with a German company to build the five-star Palace Hotel on the site of a hotel then operated by the academy.

The new hotel was subsequently completed. But no details have been published of how much money was received by the academy, nor of how the money was used. Even the organization that formally owned all the academy's property — Goskomimushchestvo — is said to have been kept in the dark.

According to a report in the newspaper *Segodnya*, government tax investigators recently visited the Palace Hotel to carry out an audit, and discovered \$860,000 in one of the hotel's safes which could not be

accounted for.

The money was apparently confiscated, but, according to the newspaper, the Prime Minister, Viktor Chernomyrdin, received a letter from the president of the academy, Yuriy Osipov, asking for its return.

Osipov: wants the money back.

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There is uncertainty in Moscow about how far the issue will be pursued. An investigation into the origins of the money was launched by Yegor Gaidar, the former first deputy prime minister and leading reformer. But following his resignation, the consequence of the poor showing of reformers in the recent elections, it will now be up to Chernomyrdin — who is on close terms with Osipov — to decide what to do next.

Vladimir Pokrovsky

Italians demand a radical reform of grant evaluation

Munich. More than 60 Italian biomedical scientists have sent a joint petition to the charities that sponsor biomedical research urging them to introduce a fairer system for allocating research funds, and in particular to adopt internationally recognized standards for evaluating grant applications.

Many of those who have signed the petition say they hope that the introduction of such changes by charities could influence state funding bodies to adopt the same procedures, but that the independent status of the charities should make it easier for them to make the first move.

At present, charities tend to follow the same assessment procedures as the National Research Council (CNR), whose 15 committees, each made up of between 10 and 20 scientists, decide which projects to fund, but are under no obligation to consult outside experts.

Many critics claim that this procedure allows decisions to be excessively influenced by personal relationships, and encourages money to be spread relatively thinly across too many research groups in order to keep everyone happy. They also criticize the CNR for its lack of transparency, as the council does not always specify the criteria on which funding decisions are made, nor does it publish a list of grants awarded.

Giovanni Romeo, professor of molecular genetics at the Giannina Gaslini Institute in Genoa, who is leading the campaign to introduce a more open refereeing system, says that charities do not have to follow the CNR's example just because it is familiar.

The petition signed by Romeo and others makes three demands: that all grant applications should be peer reviewed by independent referees, 51 per cent coming from outside Italy; that the distribution of funds should be made public, with scientific reports published at the end of the funding period; and that the members of scientific committees should be elected for a non-renewable five-year term.

Italy's two main charities for funding biomedical research are the Associazione Italiana per la Ricerca sul Cancro (AIRC, the Italian cancer research association) and Telethon, whose television appeals fund research into genetic — and particularly neuromuscular — disorders. Both meet some of these criteria; in contrast, says Romeo, most smaller charities meet none of them.



Romeo: petitioner.

The AIRC, which distributed L38 billion (US\$22.8 million) last year for projects and fellowships, already publishes a list of the projects it funds. But Giuseppe Della Porta, its scientific director, says he does not agree that the charity needs to introduce compulsory peer review. He says that the AIRC scientific committee, whose 14 members are changed at irregular intervals, can make the necessary decisions on its own, and should be left to choose whether to use outside referees. An application to extend a grant goes to a single committee member, and new applications go to two.

Della Porta sees no reason for change. "We know each other, and we know the importance of certain groups," he says, adding that the main need is to rotate committee members. He is also opposed to seeking advice from foreign scientists. "Italy has enough scientists, if committee members want to use them," he says.

Telethon, which has distributed L28 billion in the three years since it was established, is more outward looking, with half of its scientific committee of ten working abroad. Each grant application goes to three members; many applications are sent to external referees.

"The petition is a recommendation, not a criticism", says Edoardo Boncinelli, head of the laboratory of the molecular biology of development at the San Raffaele Institute of Research in Milan. "Many of the charities do very good work; but it could be improved by a proper peer review system".

Boncinelli criticizes the government for not insisting that the research it funds is appropriately assessed. Before the recent resignation of the government, Umberto Colombo, the research minister, had taken steps to change this, promising that in future three per cent of each award would be used for refereeing and evaluation, and that a formal international refereeing system would be introduced for all state-funded research. Whether these changes will now be carried through is in doubt.

Enrico Garaci, head of the CNR, says he is enthusiastic about Colombo's ideas. But he adds that he does not consider the introduction of compulsory peer review to be one of CNR's priorities. Frustrated Italian scientists may draw comfort from an example which proves that such a move, even in Italy, is indeed possible. Under a system introduced in 1989 by Gian Rossi of the Istituto Superiore di Sanita, the Italian AIDS project, which receives L30 billion a year from the ministry of health, operates a strict international peer review system for grant applications, and publishes detailed accounts every year.

Alison Abbott

Ariane still riding high despite launch failure

Kourou. Arianespace, the French space agency (CNES) and the European Space Agency (ESA) have launched a joint investigation into the failure of the 63rd flight of the Ariane rocket which crashed into the Atlantic last week carrying two satellites insured for US\$356 million.

The early phases of the rocket's flight went smoothly after it took off from the Guiana Space Centre at 18.37 local time on 24 January. In particular, officials responsible for the launch appeared relieved when the cryogenic third stage ignited successfully 5 minutes 47 seconds after take-off; three of Ariane's five past failures occurred because the third stage did not ignite (the other two failures involved the first stage).

But instead of burning for ten minutes, the stage stopped after just 80 seconds, at a height of 215 km. The rocket's ascending trajectory displayed on screens in the Jupiter control room took on an alarming downward path, and the end was inevitable.

The investigation should be completed within the next two weeks, and will focus on a sudden increase in temperature of a bearing of the liquid oxygen part of the liquid oxygen/hydrogen turbopump which was noticed 20 seconds before the pump apparently jammed.

The pump is one of the most susceptible items of Ariane technology. Weighing just 30 kg, it rotates at 60,000 r.p.m. on the hydrogen side and 13,000 r.p.m. on the oxygen side. Moreover, it must keep liquid hydrogen at -251°C and liquid oxygen at -184°C , while the temperature in the adjacent combustion chamber is over $1,500^{\circ}\text{C}$.

Arianespace has suspended its flight programme until it finds and corrects the cause of the accident. This may delay its plans to increase the frequency of launches to ten a year; Arianespace already has 37 launches, worth FFr 17 billion (US\$2.9 billion) on its order books up until 1997.

Insurance companies say any delay would reduce the income from successful launches, needed to offset the US\$356 million insurance claim for the lost Turksat 1-A and Eutelsat-II-5A satellites — the largest in the history of space. This represents a typical year's income for the space insurance industry, according to Faugère and Jutheau, the French insurance brokers handling the claim.

The company says that the accident has wiped out ten years of profits, and will cause premiums to increase. But the failure is unlikely to diminish customer confidence in the European launcher, which after 26 consecutive successes since its last failure in 1990 — caused by a rag left in the first stage — remains the world's most reliable launcher.

Declan Butler

Gene therapy gets double dose of screening

Tokyo. The human applications of gene therapy seem set to become the latest victim of the bureaucratic walls that separate the various ministries of Japan's central government in Tokyo.

Last week, a newly established gene therapy screening committee of Niigata University, a national university on the coast of the Japan Sea, gave its conditional approval to Japan's first non-therapeutic gene marker experiments to be carried out on humans, which may mark Japan's first step towards gene therapy.

But the experiments, which are similar to those carried out by French Anderson and Steven Rosenberg on cancer patients in the United States nearly five years ago, will probably need to be approved by two separate committees — one under the Ministry of Education, Science and Culture, and the other under the Ministry of Health and Welfare — before they can proceed. This bureaucratic duplication of effort will inevitably delay the Niigata experiments, as well as similar experiments elsewhere.

The proposed experiments will be carried out by Yoshiaki Moriyama, chief of the university's division of bone marrow transplantation, in collaboration with Genetic Therapy Inc., a US company established by French Anderson.

Human stem cells (CD34+ cells) and residual leukaemic cells in bone marrow taken from leukaemia patients in remission will be marked with the bacterial gene for neomycin resistance (Neo-R) after the bone marrow has been purged of most tumour

cells by heat treatment.

After transplantation back to the patient, the marked cells will be used to determine if any recurrence of cancer in the patients is caused by residual malignant cells in the purged marrow, or by cells in the patient. Genetic Therapy Inc. will provide the retrovirus vectors for gene marking.

The experiments will use the same marker gene as that used by Anderson and Rosenberg. Very similar gene marker experiments in bone marrow transplants have already been carried out by Malcolm Brenner at St Jude's Children's Research Hospital in the United States. But Brenner used unpurged bone marrow.

Moriyama and Niigata University followed the guidelines established last year by the Ministry of Health and Welfare in drawing up a research plan and screening the experiment. In line with the guidelines, the university also expanded its ethics committee into a larger gene therapy screening committee, with about ten members.

After eight months of deliberations, the committee gave approval for the experiments last week on condition that an application is made for each patient and their informed consent is obtained.

The university, however, comes under the jurisdiction of the Ministry of Education, Science and Culture, which has not yet established guidelines for gene therapy research, or a committee to screen applications for such research.

Furthermore, as the experiments involve clinical trials on humans, officials of the

Ministry of Health and Welfare believe that approval must also be sought from a committee of their ministry.

The health and welfare ministry is expected to announce the members of its advisory committee on gene therapy this week. There will be almost 20 members, including university professors, medical doctors, novelists, journalists and philosophers.

Moving more slowly off the mark, the education ministry has started approaching members of this committee asking them to join its own committee. The number of people in Japan with the expertise needed to pass judgement on such experiments is limited, and many observers expect there to be considerable overlap between the two committees. Nevertheless, it appears that Niigata University will probably require approval from both.

It is not clear which committee will have ultimate authority to reject or accept applications from university researchers. But, according to one scientist involved in setting up the government guidelines, the Ministry of Health and Welfare is unlikely to "embarrass" colleagues in the education ministry by rejecting an application that the education ministry has approved.

Researchers involved in gene therapy research criticize such duplication of effort as "bureaucratic nonsense". They would have far preferred the two ministries to have formed a single joint committee.

David Swinbanks

Nuclear relics are finally junked

More than 200 metres of top quality 2-metre diameter steel tubing (pictured right) is just one of the unusual items discarded by the Los Alamos National Laboratory that have found their way to Ed Grothus's nuclear salvage yard near the New Mexico laboratory.

The vacuum tubing, finished to mirror perfection on the inside, was used to direct laser beams at hydrogen isotopes in the Antares experiment, part of the laboratory's nuclear fusion research programme.

Grothus picked up the 125 tonnes of steel when the experiment failed and stored it in the car park of a disused supermarket. Several years later he sold part of the Antares stock back to the laboratory for a Strategic Defense Initiative project. The rest has since been cut up for scrap.

Grothus, a machinist at the laboratory

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for 20 years, began to buy obsolete pieces of equipment from auctions in 1969. Sometimes he sold his purchases on. But most were hoarded in anticipation of the construction of a 14-storey museum, planned as a catalogue of the nuclear age.

Unfortunately pressure from the local fire marshal and the Environmental Protection Agency have forced Grothus to sell up with the dream unrealized. □

David Parker/SPN

US abandons planned AIDS vaccine trial

Washington. The US Department of Defense (DoD) has abandoned its controversial proposal to carry out a large-scale, \$20-million trial of a gp160 therapeutic vaccine for AIDS developed by the Connecticut-based biotechnology company MicroGeneSys.

The move follows steady pressure from the National Institutes of Health (NIH), the Food and Drug Administration (FDA) and AIDS activist groups, each of which had challenged the value of the proposed single-drug trial.

Attempts to widen the trial into a comparison of different therapeutic vaccines floundered last year, and many scientists are in any case sceptical of the potential value of such vaccines (see *Nature* 363, 294; 1993).

A sum of \$20 million was added to a congressional spending bill in 1992 to pay for the trial, after intense lobbying by MicroGeneSys. But the wording of the bill allowed the money to revert to other Army AIDS research if DoD, NIH and the FDA agreed that the trial should not proceed, and this has now happened. **Colin Macilwain**

US ecological survey runs political gauntlet

Washington. The US National Biological Survey (NBS), proposed last year by the Secretary of the Interior, Bruce Babbitt, as the government's principal agency for ecological research, has run into unexpectedly strong opposition from anti-environmental forces in Congress.

A bill setting up the NBS was passed by the House of Representatives in October. But in the process it became the target of heated attacks by lawmakers representing private property owners, and similar pressure is expected when the bill is debated in the Senate within the next few months.

The NBS has been promoted by Babbitt as a non-political, rigorously scientific agency. But its opponents fear that data from a nationwide biological survey might be used to set aside large tracts of land as nature reserves.

The congressional attacks are part of a broader campaign by property owners to oppose any environmental measures that restrict land development without compensating the owners of the land by arguing that this is an unconstitutional appropriation of private property. The NBS debate in the House was the first opportunity to test this argument, which is also likely to be used against the Endangered Species Act, as well as legislation to protect wetlands.

An amendment to the House NBS bill proposed by Billy Tauzin (Democrat, Loui-

siana) would have required the government to compensate any property owner whose land lost value as a result of findings by the NBS.

The amendment was defeated, but only by the use of a legislative point of order that ruled it to be irrelevant to the debate. There is no such rule in the Senate, so this argument will almost certainly arise again there. "Clearly it's going to be a major fight," says Bruce Stein of the Nature Conservancy.

House opponents did succeed in passing an amendment requiring NBS employees or others working for the survey to obtain written permission from all property owners before conducting research on their land.

The standard procedure for this kind of fieldwork in the past has been to respect any existing state trespass laws. The new requirement will not prevent NBS from doing its research, says Stein, but it will significantly slow down the survey's ability to do work on private lands.

A more serious obstruction to the agency's work would be another amendment passed by the House which prohibits the NBS from accepting the services of volunteers. Biological surveys traditionally depend on fieldwork conducted not only by university researchers and museums, but

Florida's Everglades: short-listed for study.

also by conservation groups and amateur naturalists.

A report last year by the National Research Council recommended that the NBS conduct its mission in partnership with other existing groups. Peter Raven of the Missouri Botanical Garden in St Louis, who chaired the NRC study, describes the prohibition against using volunteers as "strange", and adds: "I can't see any way that it could be operational."

If the amendment is allowed by the Senate to stand, the NBS "would be the only community effort in the United States that prohibited the use of volunteers," says Raven. NBS supporters are optimistic that the House amendment will be overturned — or at least watered down — in the final legislation officially authorizing the existence of the new agency.

So far, the lack of such legislation has no real impact on the work of the NBS. The agency established by Babbitt's order last November has received \$163 million from Congress for this year's operations.

A new director, expected to be Ronald Pulliam of the University of Georgia, a former president of the Ecological Society of America, should be in post soon. The staff is already short-listing areas for the first pilot studies, which will include the Florida Everglades and other regions of ecological and political interest.

But without the authorizing legislation, continued funding for the NBS — and its very existence — are precarious. Congress appropriated money this year on the understanding that such legislation would soon be passed, and as one congressional staff member says, "you don't like to fund money for things that aren't authorized."

Senate hearings on the NBS should begin this spring, and supporters hope that final legislation will pass this summer, before the next funding cycle begins.

In the meantime, says Thomas Lovejoy of the Smithsonian Institution, who advised Babbitt on setting up the new agency, "what all of us would like to do is just build a record with the survey showing that it's not a threat."

Tony Reichhardt

Audit confirms big is not always beautiful

London. The effectiveness of Britain's investment in wind energy research has been undermined by the influence of a single potential customer, the Central Electricity Generating Board (CEGB), according to a report published last week by the National Audit Office (NAO), the government watchdog.

The CEGB's anticipated needs appeared to favour large-scale megawatt size machines as the most practical options. But social demand — as the late economist E. F. Schumacher predicted in his book *Small is Beautiful* — has proved greater for medium- and small-scale turbines.

One result appears to be that only about 20 per cent of the wind turbines installed in Britain are manufactured domestically, and exports are also small. In comparison, all wind turbines installed on Danish wind farms are manufactured in Denmark, and 60 per cent of those in the United States are built by US manufacturers.

Up to 1987, the main focus of funding for research and development was on large-scale machines. Projects covered by such funding included the construction of one of the world's largest wind turbines, a 3-MW machine built on the island of Orkney which

started operation in 1987.

In practice, however, large-scale machines have proved to be uneconomic, and the market for them (even from electricity utilities) has remained small in comparison to that for medium-sized and small wind turbine machines.

The former have been the main focus of the Department of Energy's wind energy programme only since 1987; and according to the NAO report, appeals for funding from a number of small wind turbine manufacturers serving niche markets in the United Kingdom have been virtually ignored.

Department officials told NAO investigators that they considered support for such initiatives would be more appropriate from programmes designed to promote small businesses.

Overall, the NAO report says that the department's methods for assessing the potential of different renewable energy sources is "soundly based".

But it also concludes that the earlier influence of the nationalized electricity industry as the main customer "led to a few projects and programmes receiving a large share of the £340 million spent". □

Boston cardiologist faces charges of embezzlement

Boston. A leading heart researcher at the Harvard Medical School and former chief of cardiology at the Boston Children's Hospital, who had surprised his colleagues with a lifestyle that included the purchase of an impressive collection of modern art, was last week indicted by a grand jury on six counts of embezzlement.

The indictments, which were announced by Scott Harshbarger, the attorney-general of Massachusetts, accuse Bernardo Nadal-Ginard of misappropriating \$130,000 from the Boston Children's Heart Foundation, a non-profit cardiology group practice affiliated with the Children's Hospital.

Nadal-Ginard has been charged with diverting money from the foundation — for whom he had previously served as both president and treasurer — into his personal bank accounts on six separate occasions during 1992.

"The criminal laws must be used to root out white collar fraud at all levels, regardless of one's position or socio-economic status" said Harshbarger in a press statement. "This defendant allegedly violated his position of trust and authority to steal from charitable institutions dedicated to providing important medical care."

The investigation leading to the indictments began last October after both the hospital and the foundation had notified Harshbarger's office of "financial irregularities". In November, Nadal-Ginard was placed on unpaid leave by both the medical school and the hospital, which is a Harvard teaching hospital.

He was dismissed from the hospital, where he directed a laboratory with a staff of 60 scientists and technicians, on 31 December. Carol Weinrib, the vice-president of the hospital, says that the future of those employed in the laboratory is uncertain, but that an "orderly transition" is being planned.

If convicted, the cardiologist faces a maximum sentence of five years in a state prison and a fine of \$25,000 on each embezzlement charge. The spokesperson refused to comment on what Nadal-Ginard may have done with the money he is accused of stealing, although sources close to the case claim that much of it was used to enlarge his art collection.

Nadal-Ginard is already the subject of a separate suit which was filed last November, by his former colleagues at the Children's Heart Foundation. These claim that he received "unreasonable and excessive compensation" by withdrawing more than \$4 million from a pension fund and drawing a salary of more than \$500,000, considerably in excess of the limit allowed under the rules of Harvard Medical School. **Steve Nadis**

Conflict-of-interest debate stirs mixed reaction at NIH

Washington. The proposed sponsored research agreement between Scripps Research Institute and the Sandoz drugs company, which provoked widespread controversy when it was announced last year, was anomalous and is unlikely to be replicated elsewhere, according to Sandy Chamblee, acting deputy director of the US National Institutes of Health (NIH).

But whether the problems caused by such sponsorship agreements are real (as the media tend to suggest) or not (as many scientists would claim), Congress told NIH to fix them after hearings last June before a subcommittee chaired by Ron Wyden

payer is not being fleeced by agreements that give too much away to the industrial sponsors of other research projects carried out at these institutions.

Items that have generated concern include excessively broad agreements that encompass all areas of research, deals that exclude rival companies from access to unused ideas and provisions that restrict the freedom of researchers to publish and discuss their work.

The problem, as panel member Robert Merges, professor of law at Boston University, points out, is that NIH was not set up as a regulatory body, but is having to devise a policy that is both effective and simple to administer. "It's a tough goal," says Merges.

There is a range of views on how tight the guidelines need to be. Some advocate minimal interference. "We are doing this because someone asked us to, not because there's really an issue out there," says Donald Drakeman, chief executive of Medarex and an industrialist advising the panel.

But Paul Berg, professor of biochemistry at Stanford Medical Center, claimed that the current system is destroying pure, discovery-based science in US universities. "We sit here and talk about feeding ideas into American industry, but we ignore the price we will pay," he said.

Despite Berg's complaints, the predominant view on the NIH panel was that technology transfer under Bayh-Dole has been a big success, only temporarily blemished by the Scripps-Sandoz episode. But Congressman Wyden's subcommittee still expects NIH to provide some firm conflict-of-interest rules for universities.

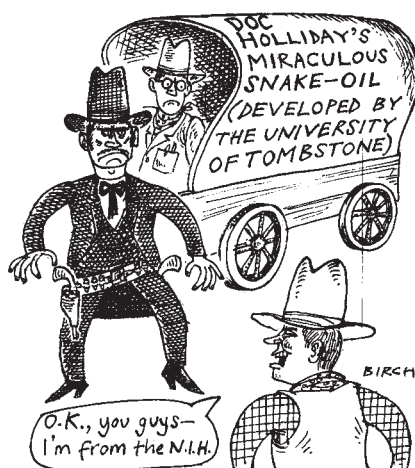
Last week's panel discussion will lead to a report for an NIH internal task force led by Chamblee, which will in turn prepare draft guidelines for Harold Varmus, the director of NIH, to take to his own advisory committee's next meeting in June.

The guidelines are expected to include a set of thresholds above which the university must obtain the approval of the NIH. Typical suggestions are that such approval should be sought for agreements worth more than \$5 million, or involving more than one fifth of the work funded by NIH at a given institution.

Universities would retain responsibility for the details of smaller deals. But they would be reminded of their obligation to protect the academic freedom of their staff.

NIH hopes that by addressing the issue publicly, it can help to dispel uncomfortable memories of the Scripps-Sandoz affair, and show that most sponsored research agreements are working in the public interest.

Colin Macilwain



(Democrat, Oregon). And the NIH is now taking steps to do so.

The \$300-million agreement between Scripps and Sandoz was effectively blocked by NIH as a result of public concern that it would have given the company excessive control over the results of federally funded research.

But Chamblee told a meeting in Bethesda, Maryland last week of a panel convened to advise NIH on the future regulation of such agreements that a survey of 375 sponsored research deals across a hundred leading US research institutions found none of the others remotely close in either scale or scope to that proposed between Scripps and Sandoz.

Many scientists and industrialists involved in sponsored research agreements believe that most of them work to the public good. Universities have been actively encouraged to transfer technology to the private sector since the passage of the Bayh-Dole act of 1980, which set the current framework for such agreements.

But, as the main source of public funds for biomedical research in the United States, the NIH pays for most work done at Scripps (and elsewhere). Congress — not unreasonably — wants NIH to ensure that the tax-

Glaxo goes back to basics in bid to meet new goals

London. The British pharmaceutical company Glaxo recently recruited Sir Mark Richmond, the former chairman of Britain's Science and Engineering Research Council (SERC) to direct its research. The appointment raised eyebrows among those who thought he would return quietly to academic life. But Richmond is quick to point out that Glaxo faces remarkably similar challenges to those he confronted at the SERC.

The company spends more than £800 million (US\$1.2 billion) a year on research and development (R&D), more than any other British company. But like drug companies worldwide, this high level of spending on research is coming increasingly under the scrutiny of investors. Glaxo's current task is to focus its research on areas most likely to provide a basic understanding of the biological processes on which its future prosperity depends.

Richmond has joined Glaxo just as Sir Richard Sykes, the company's former research director who became chief executive officer last year, has launched a "back to basics" campaign to prepare Glaxo for the future. One of Sykes's first actions has been to reinforce Glaxo's image as a research-driven company with a pipeline of innovative products, distancing himself from reports that the company was planning to diversify into generic and over-the-counter drugs.

At a time when patents on some of Glaxo's most profitable drugs are due to expire, Sykes sees this image as helping to inspire confidence among investors. "If we stick to what we are good at, there must be a customer base out there which is enormous and growing," says Sykes. "I firmly believe we are back on track."

Sykes' renewed emphasis on science is more than just window-dressing. He has, for example, separated management responsibility for research and for development. Some have interpreted this as a means of protecting the company's science base, given the prospect that institutional investors may seek cuts in R&D spending. But Sykes says his goal is "to send a clear message that medical research is very critical to the future of this organization". He adds: "These activities are so different that no one person can take care of both."

Sykes also confirms his desire to incorporate recent advances in molecular and cell biology into the base of Glaxo's R&D strat-

egy. Like other pharmaceutical companies, he says that Glaxo is less interested in the immediate gains to be made from recombinant drugs than in developing an understanding of the biological basis of chronic diseases that will lead to new therapeutics in the long term. "We are not putting a 'for sale' notice over our chemistry departments," says Richmond. "But there is a clear shift in balance between chemistry and biology."

Some critics claim that Glaxo is moving too slowly in this direction. Frustration with the company's cautious approach to radical new genome-based ventures was one reason why its director of biotechnology, Tim Harris, recently left to join Sequana Therapeutics, a small US gene therapy company based in California.

Sykes argues that, at least in the foreseeable future, gene therapy will have limited applications and markets, and that developing therapeutics for the more common chronic diseases will demand a better understanding of the complex genetic and environmental factors involved. "We don't wish to become a major gene therapy company at this stage," says Sykes. "We need to be involved, but also to ensure that we do not run off at a tangent," says Sykes.

But he also accepts that Glaxo was relatively slow to take up genetic engineering; it eventually had to buy the Swiss operations of the US biotechnology company Biogen as its entry ticket. With this in mind, the company now seeks strategic alliances with small drug development companies to keep abreast of various leading-edge innovations.

Similarly, Glaxo is turning more than in the past to university groups to keep up with relevant fundamental research. The pharmaceutical industry is now "much more openly interactive [with the basic research community]", he says.

Richmond, is likely to play a central role in steering Glaxo's interests through the thorny political debates over the extent to which the biomedical research community should be harnessed to the goals of the pharmaceutical industry.

Sykes, for example, believes that British charitable trusts need to be more commercially minded about exploiting research they fund, arguing that many British charities "have no clear mechanism for dealing with their results".

As a member of the government's new Council for Science and Technology, Sykes is well placed to ensure that his views are closely listened to in Whitehall. As chief executive of one of Britain's most research-oriented companies, his success in putting the government's ideas into practice will be closely watched.

David Dickson

Weak evidence for human link to BSE infected meat

London. Despite scientists' calls for caution, Britain's media last week gave extensive coverage to a 16-year-old girl thought to be suffering from Creutzfeldt-Jakob disease (CJD), the human equivalent of bovine spongiform encephalopathy (BSE).

Speculation that the girl might have contracted the disease through eating infected meat — in particular hamburgers — was not dampened by the fact that all attempts to find evidence that the disease can be transferred to humans in this way have drawn a blank.

Of the 50 new cases of CJD reported each year in the United Kingdom, about 15 per cent are thought to be result from inherited mutations in the gene implicated in CJD. Most of the remaining cases have no known cause, and are termed "sporadic". The 16-year-old girl, Vicky Rimmer, is thought to be such a case, although at present doctors say that CJD is only a "possible" diagnosis.

Richard Lacey, a microbiologist at Leeds University, says he is "certain" of the link between BSE and CJD in Rimmer's case, citing various "significant" factors such as the fact that 16 is a very young age to contract the disease.

But there have been at least two reported cases of CJD in teenagers, one aged 16 and the other 19. Both were sporadic cases, and are described by Robert Will, head of the CJD Surveillance Unit at Edinburgh, as "absolutely exceptional".

Other evidence used by Lacey to back his claim that BSE can be transmitted to humans is the doubling of known cases of CJD since the start of the current scare and two reported cases of farmers who had been in contact with BSE-infected cattle. The first case was published in *The Lancet* in 1992, and the second came to light last year.

"When we identified the second farmer, we felt it was something we had to investigate," says Will. Through European colleagues working on similar studies, he found one case of CJD in Belgium in 1991, two in France in 1992 and three in 1993.

But Belgium has never had BSE, and the few cases of BSE in France occurred in very different regions of the country from the instances of CJD. Will says this may show a link between farming and CJD — but nothing else.

Many scientists now fear that, as new research is published, newspapers will jump on unexpected results and publish alarmist reports. "The media are entitled to write what they want; I am a great believer in the freedom of the press," says Will. "But it is often forgotten that alarmist reports can cause a great deal of distress. Even so, we are determined to ensure that everything we find is put in the public domain." **Fiona Gammie**



Sykes: clear message.

Charges fly over rival leprosy vaccines

New Delhi. The Indian government is trying to resolve a bitter dispute between two leading scientists over the source of a leprosy vaccine in trials being conducted in northern Indian states by the National Institute of Immunology (NII) in New Delhi.

G. P. Talwar, emeritus scientist at NII, claims that the vaccine was prepared from an organism isolated in 1978 at the biochemistry laboratory, of which he was then head, of the All-India Institute of Medical Sciences (AIIMS). The organism, *Mycobacterium-w* was taken from a patient suspected of having tuberculosis, and details were published in the journal *Leprosy India* later that year.

But Mahadev Deo, director of Bombay's Cancer Research Institute — previously known as the Indian Cancer Research Centre (ICRC) — and a former student of Talwar, says that *M.w.* is identical to a bacillus cultivated in his own laboratory since 1958. A leprosy vaccine prepared from ICRC bacilli has been under trial by Deo in Maharashtra state since 1987, with results similar to those obtained with the NII vaccine.

Deo believes a mix-up could have occurred in Talwar's laboratory at the AIIMS during a study of the comparative immunology of various mycobacteria, including the ICRC bacilli supplied by the Bombay institute. He claims that the identity of the two organisms has been confirmed by a comparative study of their genomic DNA by Barry Bloom and his colleagues at the Albert Einstein College of Medicine in New York in 1989.

India's Department of Biotechnology is now investigating Deo's claim that the two vaccines are identical. The dispute has been simmering for several years; but it has been

is still a matter of dispute, and claiming that the change is being sought by individuals who, he says, had no involvement with the original isolation of the organism, as Talwar was not an author of the 1978 paper to *Leprosy India*.

Deo also alleges that an attempt is being made to "immortalize" Talwar by calling the organism *pranii*, as Talwar is known to his friends and colleagues as "pran". In response, Talwar says that a nucleotide analysis at the NII has shown that *M.w.* is "a totally new mycobacterium". He also says that the name *pranii* was chosen because the first three letters are the initials of first two authors of the paper sent to *IJL*, and the last three stand for the institute itself.

Deo's allegations are to be considered shortly by the Society for Scientific Values, a body that investigates cases of suspected

scientific fraud. Meanwhile V. Ramalingaswami, a former head of India's medical research who is mediating between the two scientists, has asked Talwar to withhold publication of the *IJL* paper until the source of *M.w.* is settled.

But there is still a hitch. The Department of Biotechnology has asked both Talwar and Deo to submit samples of their organisms for comparison using traditional and advanced microbiological tools. But so far Deo is refusing to cooperate, arguing that the data from Bloom and his colleagues have already established that the two organisms are identical.

Talwar describes Deo's attitude as counterproductive, and claims that India's progress in leprosy research is being threatened by what he calls unfounded statements and allegations.

K. S. Jayaraman

South Korea launches Biotech-2000

Tokyo. The government of South Korea has launched a massive government-industry programme to promote the development of biotechnology. Planned as part of a broad strategy to catch up with the technology of advanced nations, the programme will, according to the Ministry of Science and Technology (MOST), involve the expenditure of 16,000 billion won (nearly US\$20 billion) over the next 14 years.

The new programme is called Biotech-2000. It began earlier this month with a government budget of 54 billion won (US\$67 million) for the current fiscal year; industry is expected to contribute another 167 billion won.

The figure of \$20 billion to be spent in the period up to 2007 needs to be treated with a certain amount of scepticism, however. When South Korea launched its 'G-7 project' in 1991 to catch up with the technological achievements of leading industrial nations in fields such as computer memory chips, the government estimated that total investment would be about US\$7 billion by 2001.

By last year this estimate had dropped to \$4.2 billion, with actual expenditure in the first two years of the ten-year project amounting to about 550 billion won (US\$700 million) (see *Nature* 364, 382; 1993). One diplomat in Seoul says that he always divides such projections by three to arrive at a more realistic estimate.

But despite such scepticism, Biotech-2000 is still likely to be big, and will dwarf the G-7 project in scale. As with G-7, several other ministries will participate in addition to MOST. These include the Ministry of Trade, Industry and Energy, the Ministry of Health and Social Affairs, the Ministry of

Education, the Ministry of the Environment and the Ministry of Agriculture, Forestry and Fisheries.

The programme will cover six broad fields: industrial technology; health technology (including biomedical engineering, molecular biology of biological functions, and human genome research); agricultural and food technology; environmental technology; energy technology; and basic life science technology.

The key participants will be MOST's Genetic Engineering Research Institute (GERI) in Taeduck science town, and the agricultural ministry's Korea Food Research Institute in Seoul, as well as the Korea Institute of Science and Technology (KIST), to which GERI is affiliated.

Under its 'centre of excellence' scheme, MOST will also provide about \$1 million a year over nine years to nine selected centres in universities that will participate in the programme.

Biotech-2000 was first proposed by GERI in 1991, according to Hyu Gyu Lee, director of MOST's research planning division. But it did not get off the ground until last July, when it received the backing of a recently-formed association of biotechnology academics and industrialists, says a GERI official.

The minister of science and technology, Si Joong Kim, appointed last year and a chemist by training, has been a major driving force behind the biotechnology programme. Bioscientists expect it to prosper as long as he remains in power. But changes in ministerial posts in South Korea are frequent, and the long-term future of the programme therefore remains uncertain.

David Swinbanks

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Shawn Sprague/Photos Pictures

Leprosy treatment in a Bombay clinic.

brought to a head by an attempt by Talwar and his three colleagues at the NII to change the name of the organism from *M.w.* to *M.pranii*.

The change of name has been proposed in a paper submitted to the *International Journal of Leprosy (IJL)*. But in a letter to the Department of Biotechnology, Deo describes the proposed renaming as unethical and a "fraud on the scientific community", pointing out that the source of the organism

Women's health and diet

SIR — On behalf of the investigators responsible for mounting the NIH Women's Health Initiative (WHI), we wish to comment on the article (*Nature* 366, 11; 1993) summarizing the recent assessment of WHI by a committee appointed by the Institute of Medicine.

The committee focused primarily on the clinical trial, one of three components of WHI, which is already under way at 16 US clinical centres.

The clinical trial is designed to measure the effects of hormone replacement therapy, dietary modification and calcium and vitamin-D supplementation on the overall health of postmenopausal women. All three treatments are already widely used, and each is presumed to have major public health implications. We believe that it is essential to obtain reliable and up-to-date information on the benefits and risks of these interventions in the middle and later decades of women's lives.

Although the committee was critical of some aspects of the clinical trial, it recommended that each component of it should continue, and confirmed that the budget was not excessive — hardly a "thorough condemnation of a study designed by fellow professionals".

The article implies that WHI is a "hastily put together programme", ignoring our work in successful preliminary studies among large numbers of postmenopausal women over the past decade. In particular, these studies have led to the development of a practical, successful programme for adopting a low-fat eating pattern.

On the dietary modification part of the clinical trial, the committee reported that its members support hypotheses relating a low fat-eating pattern to a reduced risk of coronary heart disease and of colorectal cancer, but regard the data supporting a reduced risk of breast cancer as weak. In our view, except for the link between dietary factors and blood cholesterol, evidence relating dietary fat to coronary heart disease is virtually identical to that for breast cancer. Specifically, analyses of variations in international disease rates, of national time trends in disease rates and of the experience of migrant populations support the existence of relationships for both coronary heart disease and breast cancer, whereas case control and cohort studies yield inconsistent results for both. Furthermore, fundamental problems in assessing the diets of individuals make it unlikely that additional case-control and cohort studies alone can reliably answer whether or not a dietary change can reduce the risk of breast cancer.

The article correctly reports the committee's concern about aspects of the WHI statistical design, citing specifically an

assumption of a linear halving of breast cancer risk over five years among women who adhere to the dietary change programme as compared to women who make no dietary change. In fact, this is one place where the committee misunderstood our 147-page protocol. The actual design assumption assumed risk to be halved over a ten- rather than a five-year period. Careful allowances for lack of adherence to dietary goals, secular trends in control group dietary habits and deaths from competing risks then lead to a projected 14 per cent lower breast cancer risk for dietary intervention group as compared to control group women.

Brief responses to the other design issues are as follows. We believe it is important to establish whether or not a combination of calcium and vitamin D can prevent hip and other fractures, and less immediate to know the relative contributions of each to any preventive success; we believe that it is important to have some study of the effects, for example, in respect to quality of life and safety, of undertaking both hormone replacement therapy and a low-fat eating pattern, but that a statistically powerful test for "interaction between the two regimens" is less important. As the treatments to be assessed are already being widely adopted, we believe that testing in the general population, rather than just in women thought to be at high risk for selected diseases, is needed, and clinical-trial sample sizes have been chosen accordingly.

The consent forms being used in the WHI have undergone a considerable process of standardization, review and approval, including review and approval by our external Data and Safety Monitoring Board and by the Investigational Review Boards of each of our institutions. But we welcome the committee's encouragement to strive for the highest standard for communicating possible risks and benefits. To this end we are systematically examining all our consent procedures with a view to adding more quantitative information while avoiding information overload on participants.

Finally, as you report, the committee was concerned that the current budget allocations may be insufficient for this ambitious programme. However, we believe that the average 9-year follow-up period in the WHI clinical trial with lower costs in later years, and the design efficiencies resulting from cost-assessment components of the extensive feasibility and pilot studies, will allow the WHI clinical trial to continue in a cost-efficient manner. In fact, our own cost projections indicate that the project can be completed within the planned budget.

In summary, we appreciate the committee's thoughtful recommendations, including that of a formal clinical trial assessment at an earlier date than had been intended, which we do not regard as 'condemnation' but as encouragement to conduct the strongest, most efficient study possible.

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SIR — In her article on the NIH women's study, Barbara J. Culliton mentions osteoporosis and the complications of calcium intake. Studies of the influence of vitamin D on the occurrence and severity of osteoporosis in white women should be possible by comparing groups of postmenopausal white women living in a temperate climate with a lack of winter sunlight and those living in a sunny tropical climate.

On a small scale (60 participants), we made such study two years ago in the Netherlands and in Curaçao in the Netherlands Antilles (*Am. J. clin. Nutr.* 58, 106; 1993). The calcium intake in the Curaçao women was only moderately lower than in the Dutch group, so that the vitamin-D synthesis in the skin of both groups is most probably the chief influence on bone density. We estimate the cost of the published part of our investigation at less than £35,000.

The small size of the white population of Curaçao makes an investigation on a larger scale desirable. The participation of a few hundred white women living in a temperate and in a tropical climate might clarify the phenomenon of periodic (winter) vitamin-D deficiency in postmenopausal women.

If organized, such an investigation need take at most two years. The cost might be higher than in thrifty Holland, but should be less than £300,000.

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More global treaties

SIR — Global environmental policies will be ineffective without international cooperation. As part of a study of multilateral environmental accords from 1920 to 1990, we examined the record of multilateral treaty commitments by several large countries, looking at the total number of treaties legally joined by each and at whether the treaties: (1) constrained the domestic activities and sovereignty of the signatory; (2) addressed problems of global commons (as opposed to territorial problems); and (3) instituted regimes requiring active collaboration. We find important similarities (and differences) in treaty-making patterns, suggesting that norms and practices are shared to a greater extent than is commonly believed.

The top part of the table shows the treaty-making patterns of four 'advanced' countries, with a combined gross national product amounting to nearly 60 per cent of the global total. Perhaps because of its coastlines along both the North Sea and the Baltic, as well as its position in the centre of Europe, Germany participates in more environmental treaties (53) than the others, and shows the greatest tendency to accept constraints on domestic activities (33 treaties, or 62 per cent). More surprising is the extensive participation of the former Soviet Union, which has signed more treaties than the United States and Japan, and has accepted domestic sovereignty constraints in half of them.

The main industrial countries were rather uniform in their participation in commons and collaboration treaties. The United States exhibits the highest percentage of each type, but only by a small margin. Japan participates in only 37 treaties, 16 (43 per cent) involving con-

straints on domestic sovereignty. But these numbers may well change, given Japan's recent activism on environmental issues.

The bottom part of the table shows the treaty commitments of four large developing countries, with a combined population accounting for nearly 44 per cent of the global total. These countries show lower rates of participation than the main industrialized countries, but they are relatively uniform among themselves. Like the developed countries, these countries are almost identical in percentage of commitments that were commons and collaboration treaties. Indonesia participates in a smaller number of treaties (13) than the others in the group, but nearly 70 per cent involve domestic sovereignty. At the top of the group by a small margin, India joined 26 treaties, 65 per cent of which constrain domestic sovereignty.

Given China's status as the world's most populous country (and one of the fastest-growing economies), its participation in environmental treaties is particularly important. China ratified 22 treaties, a notable increase from the 15 it had ratified only a few years ago. Although only 10 of these involve constraints on domestic activities, China's performance is nevertheless encouraging considering its attitudes on most issues in world politics.

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PARTICIPATION IN MULTILATERAL ENVIRONMENTAL TREATIES

	United States	Japan	Germany	Soviet Union
Sovereignty-constraint				
Domestic	18 (42)	16 (43)	33 (62)	24 (50)
Foreign	25 (58)	21 (57)	20 (38)	24 (50)
Problem-type				
Commons	28 (65)	22 (59)	32 (60)	30 (63)
Territorial	15 (35)	15 (41)	21 (40)	18 (38)
Regime-type				
Common aversions	16 (37)	16 (43)	22 (42)	19 (40)
Common interests	27 (63)	21 (57)	31 (58)	29 (60)
Total treaties in force	43	37	53	48
Variable	China	Brazil	India	Indonesia
Sovereignty-constraint				
Domestic	10 (45)	14 (58)	17 (65)	9 (69)
Foreign	12 (55)	10 (42)	9 (35)	4 (31)
Problem-type				
Commons	12 (55)	11 (46)	11 (42)	6 (46)
Territorial	10 (45)	13 (54)	15 (58)	7 (54)
Regime-type				
Common aversions	11 (50)	12 (50)	14 (54)	6 (46)
Common interests	11 (50)	12 (50)	12 (46)	7 (54)
Total treaties in force	22	24	26	13

Numbers in brackets, percentages for each variable. Types of treaty are defined in the text.

Adrenocentenary

SIR — Heilbron and Bynum omitted from this list of discoveries having their centenary in 1994 (*Nature* 364, 11–14; 1994) the discovery of adrenaline, which was discovered by George Oliver and Edward Schaefer, here at University College London, in the department of physiology and first reported on 10 March 1894 (*Proc. Physiol. Soc.* 8, 1; 1894; *J. Physiol.* 16: i-v; 1895 & 17, ix-xiv; 1895).

The first hormone to be called a hormone was of course secretin, also a product of this department, but in retrospect I think all will agree that adrenaline, also a true internal secretion, can justifiably claim priority. It was the first to be assigned a chemical structure, although John J. Abel and R. de M. Taveau of Johns Hopkins University got it wrong at the first try (*J. biol. Chem.* 1, 1; 1905). It was also the first to be chemically synthesized.

The assignment of the structure of adrenaline is also notable as an early instance of scientific skulduggery. Somewhere along the line, the Parke Davis Company got in on the act. It sent Jokichi Takamine to Baltimore and, with the information he gathered, went on to solve the structure. The name Adrenaline became a registered trademark of the Parke Davis Company: hence the US use of the term epinephrine.

All interested Adrenocentenaries are invited to attend a celebratory symposium to be held at the Wellcome Trust (10 and 11 March). This has the support of the Wellcome Trust, the Biochemical Society, the British Heart Foundation and the Physiological Society. In the very best traditions, the Parke Davis Company, invited to redeem its sins, has declined to comment.

B. D. Gomperts

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HIV and AIDS

SIR — If the serious medical problems of haemophiliacs were due to a general protein contamination of factor VIII treatments, as previously prepared, then these problems would have been apparent long before the spread of HIV. There does not appear to have been such a high risk apparent in the general population before the HIV epidemic. Hence I infer that AIDS in haemophiliacs is much more consistent with the HIV theory than with a general contamination hypothesis as suggested elsewhere.

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Benefits from SAX

SIR — We feel obliged to respond to your article (*Nature* 366, 101; 1993) on the X-ray satellite SAX, which contains a number of mere statements about the competitiveness of SAX — or rather the lack of it — without context or backing.

One of the most prominent features of the X-ray sky is its extreme variability, with many transient and burst-like phenomena. To study the astrophysics of compact stellar X-ray binaries and nuclei of active galaxies for example, a consistent astrophysical picture can be obtained only by observations over the widest possible X-ray bandwidth, and with a certain spectral resolving power.

The unique feature of the SAX satellite is that it carries a scientific payload comprising a balanced set of instruments that are mutually well tuned in sensitivity and spectral resolution so as to provide simultaneity over an unprecedented combination of X-ray bandwidth (three orders of magnitude 0.2–200 keV) and spectral resolving power ($\lambda/\Delta\lambda$ typically 10–20). This is achieved by four bore-sighted X-ray (imaging) spectrometers with a typical field of view of 1° and with substantial overlap, which allows for adequate normalization of sensitivity and the cancellation of undesirable systematic effects in individual instruments. Moreover, careful selection of the SAX low-Earth (near equatorial) orbit permits operation in an environment of stable and minimal radiation-induced background noise. Many of the instruments are novel.

Second, SAX's wide-band spectral coverage will also be linked to its ability to detect and monitor highly variable and transient X-ray sources with the aid of two wide-field (35°) X-ray cameras. This provides a unique potential for dynamical studies of transient and non-periodic phenomena in the X-ray sky. The wide-field X-ray cameras on SAX, a prototype of which is still operational on the Russian space station Mir, are the most sophisticated shadow-mask cameras so far developed in terms of field coverage over angular resolving power (3×10^5 pixels) and sensitivity.

If compared to the impressive performance of the two major X-ray satellites now in operation, ROSAT and ASCA, SAX is both competitive and complementary. ROSAT now mainly focuses on deep imaging and mapping of sources with low X-ray surface brightness limited to soft X-rays (< 2.5 keV). The ASCA mission, launched this year, is directed towards medium-resolution spectroscopic studies of X-ray sources in the 0.5–10 keV pass-band, albeit with higher throughput and somewhat better energy resolution, but with lower spatial resolution than the SAX instrument covering this part of the

spectrum. SAX, on the other hand, aims primarily at attaining high-quality spectral variation data on relatively bright variable and transient sources including the high energy continuum (> 10 keV), which is beyond the reach of ROSAT and ASCA.

In summary, SAX is a sophisticated satellite with state-of-the-art instrumentation, which, with its unique combination of medium spatial and spectral resolution and broad band energy response, will provide unique insights into a wide range of astrophysical objects.

We wish to emphasize, however, that the discussion in your article of the evolution in costs of SAX is confusing without a proper reference. For external reasons, the project has undergone substantial evolution and delay since its inception in 1992, such as the transfer from a shuttle flight free of charge to a fully charged Atlas Centaur, the associated spacecraft redesign and the inclusion of a new ground segment infrastructure; moreover, the figures quoted in the article refer to economic conditions covering a period of more than ten years; without a proper backdating, comparison is meaningless.

I write on behalf of the SAX Science Steering Committee, of which I am chairman and whose members are J. A. M. Bleeker (Space Research Organization, Utrecht, The Netherlands); G. Boella (CNR Istituto Fisica Cosmica e Tecnologie Relative, Milan, Italy); G. Di Cuccio (Istituto Tecnologie e Studio Radioazioni Extraterrestri, Bologna, Italy); G. C. Perola (Istituto Osservatorio Astronomico Università 'La Sapienza', Rome, Italy); A. Preite-Martinez (CNR Istituto Astrofisica Spaziale, Frascati, Italy); M. Salvati (Osservatorio Astrofisico Arcetri, Firenze, Italy); B. G. Taylor (ESTEC/ESA, Noordwijk, The Netherlands); E. P. J. Van den Heuvel (Astronomical Institute 'A. Pannekoek', University of Amsterdam, The Netherlands).

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SIR — I basically agree with Professor Scarsi in the preceding letter about the scientific potential of SAX. This X-ray astronomy satellite will combine a number of instruments which, when taken separately, do not surpass the capabilities of current instruments on MIR-KVANT, Granat, ROSAT, GRO, ASCA and XTE. But taken together, the SAX instruments will be very useful because they combine broad energy band on the one hand with the richness and variability of the X-ray sky on the other.

The main problems of SAX are political

and managerial. The delays caused by a weak management have had a direct impact on the cost as well as on the scientific potential. The latter is being eroded simply because the frontline of X-ray astronomy is progressing. It is imperative to strengthen the project management and control in order to save SAX.

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'Wrong' ideas

SIR — Jonathan Cowie (*Nature* 365, 202; 1993), from the fact that only 5.7 per cent of authors have difficulties with publication of their work in peer-reviewed journals, drew the conclusion that most landmark works (perhaps 94.3 per cent) were and are "readily considered by the scientific community". But the number of landmark works originally rejected is too large to be explained by chance.

The problem of unorthodox ideas and work in science is becoming more acute with the growth of the institutionalization of science, with the concomitant rise of conformism and uniformity of thinking. The mechanism is simple: if a scientist is developing an idea in contradiction with accepted dogma, the chance of obtaining funds or of publishing a paper is small.

The root of the problem seems to lie in the general unconscious acceptance of the philosophy of naive realism, the basis of which is the belief that 'absolute truth' exists and is reflected, at least partially, by 'true' theories; the role of science is to distinguish the true theory from several rival ones. After the 'true' theory is selected and considered to be proved, all efforts and funds are concentrated on its development; any attempt to reconsider the 'wrong' ideas is unwelcome.

This is similar to the notorious principle of democratic centralism which was popular in totalitarian communist society: a question may be discussed, but after the collective wisdom (the majority of votes) has found the 'true' decision, any discussion of alternative views is prohibited.

There are no 'true' and 'wrong' ideas or theories in reality: there are fruitful ideas and infertile ones. The history of science shows that many ideas at first declared 'wrong' have subsequently been shown to be 'true'. The main lesson of history is that nobody can ultimately decide what is the truth and what is not. The best way to proceed is not to try to 'prove' or 'disprove' a theory, but to concentrate attention on the positive aspects of any theory. Theories should be considered not as rivals but rather as complementary.

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A new forerunner for continental drift

James Romm

Abraham Ortelius suggested the basic elements of the continental drift theory in 1596, thus antedating by more than 150 years other writers credited with early formulations of the theory.

THE theory of continental drift was first scientifically formulated by F. B. Taylor and Alfred Wegener in 1910 and 1912, respectively, but the germ of the idea has been traced back far earlier^{1,2}. An eighteenth-century theologian, Theodor Christoph Lilienthal, and the nineteenth-century geologist Antonio Snider-Pellegrini, have been named as the first forerunners of the continental drift theory. But Abraham Ortelius, in remarks which have so far gone unnoticed, expressed the same idea well before either of these men. In a discussion of Plato's Atlantis legend which he published in the third edition of his *Thesaurus Geographicus*³, a dictionary of classical place names, Ortelius suggested that Plato had described an ancient separation of the continents, and used this interpretation to account for the matching coastlines of the Old and New Worlds — a phenomenon which he may well have been first to point out.

Continental congruity

Francis Bacon was, until recently, credited with the first observation of the congruity or 'fit' of the continents: in a passage in his *Novum Organum* (1620), he wrote of the Old and New Worlds as examples of "conformable instances"⁴. However, both A. Carozzi in 1969, and N. A. Rupke a year later, noted that Bacon's point concerns the symmetry of the continents rather than their complementarity (see refs 5, 6). In fact, his failure to mention the continental fit, despite his obvious interest in the structure of the Earth's land masses, is a remarkable omission, and strongly suggests that he had not made this observation nor heard it discussed elsewhere.

Rupke⁶ also invalidates the claims that François Placet in the seventeenth century, and the Comte de Buffon in the eighteenth, noticed this fit or otherwise anticipated continental drift. Placet, a French cleric and theologian, theorized in 1668 (ref. 7) that "before the Deluge America was not separated from the other parts of the earth, and there were no islands". But, as Rupke points out, the word "separated" is misleading here, in that Placet did not envisage a breaking-off of America from Europe; rather, in his view America was first created during the upheavals that disturbed the original unity of the globe, and which also caused the subsidence of Plato's Atlantis. Thus it can

be assumed that Placet too, like Bacon, failed to perceive the congruity of the continents, for had he noticed it he could have greatly simplified and strengthened his theory. Rupke then goes on to show that Buffon, in his 1749 *Théorie de la Terre*⁸, likewise missed this congruity, and in fact claimed that Africa and South America showed opposing, not complementary, projections.

Moving continents

The first instance of a true "idea of continental drift" cited by Rupke⁶ is found in the Biblical exegesis of Theodor Christoph Lilienthal⁹, a German theologian of the mid-eighteenth century. Although it is clear that Lilienthal did perceive the continental fit, did he conceive the other essential element of the continental drift idea, that of the movement of land masses? The crucial passage comes in his interpretation of 1 Chronicles 1:19 — "And to Heber there were born two sons; the one was named Phaleg, because in his day the earth was divided". Lilienthal asks how the Earth could be called "divided" at a time when only a very few humans inhabited it, and records a solution proposed by some of his contemporaries: the Hebrew word meaning "divided" refers to the separation of lands by waters, rather than the allotment of territories to different peoples. After supporting this solu-

tion with etymological arguments, and with a citation from Pliny the elder showing that many lands have in fact been cut off from each other by sea, Lilienthal adds the following, here translated from the German:

This is also likely owing to the fact that the coasts of certain lands, situated opposite each other though separated by sea, have a corresponding shape, so that they would be congruent with one another were they to stand side by side; for example, the southern part of America and Africa. For this reason one supposes that perhaps both of these continents were previously attached to each other, either directly, or through the sunken island of Atlantis; just as Sicily was attached to Naples, Ceylon to the mainland of India, Sumatra to Malacca, Britain to France, and so on.

Lilienthal draws no clear deduction regarding the movement of the continents from his observation of their fit. By comparing the relationship of South America and Africa with that of Sicily and Italy, for example, Lilienthal implies that erosion, or perhaps changes in sea level (as befitting the Flood), had sundered these land masses — ignoring the fact that their matching coastlines would not be explained in this manner. Similarly when he says that the continents had once been connected either directly or by way of an intermediate land mass, he raises the



Map of the world by Ortelius, who noticed the congruity of coastlines, which he tried to explain as arising from a catastrophic event. (From *Theatrum Orbis Terrarum*, 1598.)

possibility that the continents had not moved apart but had been separated by the sinking of Atlantis (in which case their congruity would be nothing more than an irrelevant coincidence). In fact, the only movement of land masses suggested in the above passage is the hypothetical rejoining of the continents which Lilienthal envisions, as a way of demonstrating the closeness of their fit. Thus it is not the case that Lilienthal "conceived the possibility of continental drift", as Rupke proposed.

Carozzi⁵ identified Antonio Snider-Pellegrini as "the first forerunner of the idea of continental drift". Snider-Pellegrini¹⁰ relied on new geological evidence, including the continuity of rock formations in Africa and South America, to prove the original unity of the continents, while still using the Bible and Plato as a framework for understanding their separation. According to Snider-Pellegrini, the New World had split off from the Old during a single, violent expansion of the Earth's crust, caused by the eruption of volcanic material along a North-South fissure between the two. This catastrophic event was simultaneous with the Noachic Flood, but did not involve the subsidence or erosion of any pre-existing land masses; rather, the Flood is invoked here simply to account for the creation of the Atlantic Ocean in the growing rift between the continents. In addition to the Bible, Snider-Pellegrini applies the mythology of Plato's *Timaeus* and *Critias* to the events he describes, claiming that America is identical with the ancient island of Atlantis. Evidently he correlated the Noachic Flood with the cataclysm that, according to *Timaeus*, had sunk Atlantis, and assumed that what Plato perceived as the sinking of the island was actually a sudden movement to the west. Thus Snider-Pellegrini can certainly be credited with a rudimentary idea of continental drift, as he explicitly postulated both the original unity of the continents and their subsequent movement.

The contribution of Ortelius

But Abraham Ortelius had already postulated both these elements more than 250 years before Snider-Pellegrini, in the 1596 edition of his *Thesaurus Geographicus*¹. Ortelius was a geographer and student of classical antiquity; he published the famous atlas *Theatrum Orbis Terrarum* in 1570, and thereafter compiled his dictionary of classical toponyms, first published in 1578 under the title *Synonymia* and later renamed *Thesaurus Geographicus*. In both works, Ortelius speculated about the geographical content of Greco-Roman myths such as Plato's tale of Atlantis, which in the Renaissance was widely interpreted as a historically accurate account of the Americas. In a passage he added to the third edition of the



Abraham Ortelius, 1527–1598.

Thesaurus, Ortelius discussed this interpretation in connection with "Gadiricus", the name Plato had used in the *Critias* to refer to the easternmost portion of Atlantis — the portion which, Plato claimed, was shorn off from Atlantis during its subsidence and survived to become the island of Gadir (modern-day Cadiz). Ortelius felt that the historical accuracy of the Atlantis legend rested on whether or not the *Critias* could be reconciled with his own understanding of global structure. He reasoned as follows:

Graeci nomen EUMELI, *eumélou*, olim habuit, vernaculae linguae Gadiri appellationem referens. Ut refert Plato, in Critia, sive Atlantide. Nisi fabula sit, Gadir sive Gades pars erit reliqua Atlantidis sive Americae insulae, atque haec non tam submersa (ut idem refert in Timaeo) quam ab Europa atque Africa terrae motu et illuvione abrupta: et recta occidentem versus elongata videbitur. Quod si quis hoc Fabulam fabula compensare vocet, per me quidem licebit. Ostendunt se rupturae vestigia, si quis harum trium dictarum terrarum (adhibita geographica universalis tabula) partium littora, quo se mutuo aspiciunt, eminentiasque Europae nempe atque Africae cum concavitatibus Americae penitus consideraverit. Adeo ut quis posset cum Strabone 2 dicere, non esse figmentum quod Plato ex Solonis sententia, de Atlantis insula prodiderit.

The Greeks used to have the name Eumelus, giving a translation of the native language of Gadir, as Plato reports in the *Critias* or *Atlantis*. If this work is not a fiction, then Gadir or Gades will be the remaining part of the island Atlantis or America — which was not sunk (as Plato reports in the *Timaeus*) so much as torn away from Europe and Africa, by earthquakes and flood — and accordingly will seem to be elongated toward the West. But if someone refers to this as making good

one fiction by telling another, he has a right to do so, as far as I'm concerned. But the vestiges of the rupture reveal themselves, if someone brings forward a map of the world and considers carefully the coasts of the three aforementioned parts of the earth, where they face each other — I mean the projecting parts of Europe and Africa, of course, along with the recesses of America. The case is such that one might say, along with Book 2 of Strabo, that what Plato related concerning Atlantis, on the authority of Solon, was not an invention.

Here, Ortelius made two remarkable breakthroughs in scientific thought, anticipating those of Lilienthal and Snider-Pellegrini. First, he noted the congruity of coastlines of the Old and New Worlds, perhaps as a result of his own work on the world map of his 1570 atlas, *Theatrum Orbis Terrarum*. Second, he has tried to envision a catastrophic event that could have moved the continents apart, turning, as Snider-Pellegrini was to do, to the Atlantis flood of the *Timaeus* (but without tying it to the Biblical flood, or explaining either event in geological terms). If Plato's *Timaeus* is to be believed, Ortelius suggested, it must be reinterpreted to reflect the dislocation, rather than the subsidence, of Atlantis/America; once this is accomplished, the congruity of the New and Old Worlds makes historical sense.

One can only speculate as to why such prescient remarks went unnoticed by Ortelius' contemporaries, so that even the fundamental observation regarding the fit of the continents eluded both Bacon and Placet and was rediscovered only in the mid-eighteenth century. Ortelius may have felt some timidity about publicizing his speculations, as by incorporating them into only a single entry of a vast reference work he helped ensure their obscurity. Only now, nearly four centuries after its initial publication, does this remarkable passage take its rightful place in the history of the continental drift theory. □

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Origin of life by careful reading

A small meeting last week on the origin of life pinned down some important questions and showed how many more remain unanswered; a more systematic search of the literature for clues would help.

ON the reductionists' agenda, the origin of terrestrial life necessarily has a central place. If the nature of the world we live in is determined simply by its constituents and the laws of physics, a demonstration that it is possible for self-replicating organisms to arise spontaneously may be the only way of countering the common scepticism that nothing as marvellous as, say, the intricacies of the human eye (let alone brain) could have arisen "by chance" — the derogatory shorthand for "evolution by natural selection". How well are the reductionists doing?

A prior caveat is important: to keep scepticism at bay, the reductionists do not have to recreate life as it exists on the Earth. It would suffice to show that organisms capable of replicating themselves could plausibly have arisen from *primaeval* inorganic matter. Indeed, it would then be possible to proclaim that our form of life is merely one of several, which is probably the case.

That, of course, is not a novel thought. More than 30 years ago, the late Zdenek Kopal, in his inaugural lecture as the University of Manchester's first professor of astronomy, committed himself to some speculations about the likelihood that radioastronomers would find evidence of person-like life elsewhere and stimulated from Dr Thomas Gold the memorable stage-whisper, "Bloody fool, they might be like rocks!", which was meant as a reference to the place of silicon as the next member after carbon in Group IV of the Periodic Table.

But reconstructing the origin of forms of life not based on carbon must be a much more difficult (or, certainly, a more imaginative) task than that of working out the history of that with which we are increasingly familiar. Indeed, with a little luck, the history may already be written in the genomes of the organisms now extant. For at least three-quarters of the time since molecular biology came of age, people have been remarking on the way in which particular sequences of amino acids and nucleotides are conserved from one species to another, suggesting that even small changes have fallen foul of natural selection and thus that the function of the sequences is *primaeval*. When it is possible to compare the entire genome sequences of very different organisms and, in particular, to understand the function of the "junk" DNA they carry, who will be surprised if the junk proves to be at least a partial record of past evolution?

Meanwhile, present clues to past events are still painfully sparse. So much was apparent from last week's meeting at the Ciba Foundation in London, held on the back of the Royal Society's meeting on planetary science and therefore given to marvelling at the formation of hydrocarbons or their chemical derivatives in various kinds of molecular clouds. Is that where our life began? Sir Fred Hoyle might like to think so, but panspermia remains an implausible hypothesis, and (by Occam's razor) a need-

less one as well. Two independent strands of evidence do however pin down the interval during which life began on the surface of the Earth. The dramatic proof provided by the Apollo rocks of major meteoritic impacts on the surface of the Moon between 4.1 billion and 3.9 billion years ago, mirrored by less direct proof that Mercury and Mars suffered similarly, suggests that the Earth must also have been sterile by the time the bombardment ended. But palaeontologists who have learned to recognize agglomerations of single-celled organisms in Archaean rocks are confident that life of some kind was flourishing as quickly as between 200 and 400 million years later. By the uncertainties that attend such ancient dates, that is almost like having an age for the origin of life.

An interesting side-issue arose briefly last week: if the Earth was populated with life so soon after it had been meteoritically sterilized, why did three billion years then go by before fossils of creatures with differently specialized cells began appearing in the fossil record, whereupon there was a huge explosion of diversity? That objection can be met in two ways. First, there was a lot going on. Single-celled organisms can be as complicated as jellyfish, slime moulds and fungi. As someone put it last week, "they had to learn all that chemistry". But who, in any case, says that the acquisition of the biochemical machinery of differentiation and sexual reproduction, the origin of biodiversity, would have been child's play?

So what were the first forms of life really like? As yet, nobody can know. In the half century since Harold Urey and Stanley Miller at the University of Chicago sparked electric discharges through mixtures of water, hydrogen, methane and ammonia (to simulate lightning in the *primaeval* atmosphere) and made small quantities of amino acids and purines, it has been reasonable to suppose that at least the essential building blocks of life would have been accessible. But the first self-replicating entities must have been molecules, not organisms in the ordinary sense. To escape the dead hand of equilib-

rium thermodynamics, they must have been autocatalytic molecules.

That is why the discovery (in the 1970s) of catalysis by molecules of RNA has been such a stimulus for those working in the field. Indeed, the first self-catalysed replicating system, due to Gunter von Kiedrowski in 1986, consisted of the linkage of two trinucleotide molecules catalysed by the hexanucleotide that is the product of the linkage. This is what Leslie Orgel has called "informational" autocatalysis (see *Nature* **358**, 203; 1992), but he also explains why it is unlikely to be extendable to longer RNA molecules; the template and its product would not dissociate.

With all that said, the literature these days seems full of pointers to what life may have been like soon after it began. Thus it is an article of faith that the activity of modern genes is regulated by the interaction between proteins and the relevant DNA, which prompts the question of the *primaeval* role of these interactions. Then there are ribosomes, the repository of more than 90 per cent of the RNA in a typical cell, which now regulate the translation of nucleotides, three units at a time, into protein residues; what were their *primaeval* functions?

Several articles in this issue of *Nature*, as in almost any other, may be clues to what *primaeval* life was like. Quite apart from the self-assembling double helix organized around sodium ions (page 441) and the interactions between the genes called *even-skipped* and *wingless* and between *wingless* and *engrailed* (pages 429 and 460) in several insect orders (all long post-*primaeval*), the tentative structure of the *Drosophila* transcription factor (page 484), with its six component protein elements, has an Archaean ring to it, especially when account is taken of similarities with the notoriously well-conserved histone proteins. The touch-sensitive sodium channel found in the nematode *Caenorhabditis elegans*, also post-*primaeval*, like the analogous epithelial sodium channel in the rat kidney, while nicely suggesting how natural selection has led to the formation of a more efficient channel by triplication of a pre-existing gene (pages 463, 467 and 470), is similarly a reminder of the need that the invention of membrane channels should have followed on the heels of that of membranes themselves. Perhaps the next big effort, in the search for the origin of life, should be a careful reading of the journals in the hope of defining the latest dates at which such features could have arisen.

John Maddox

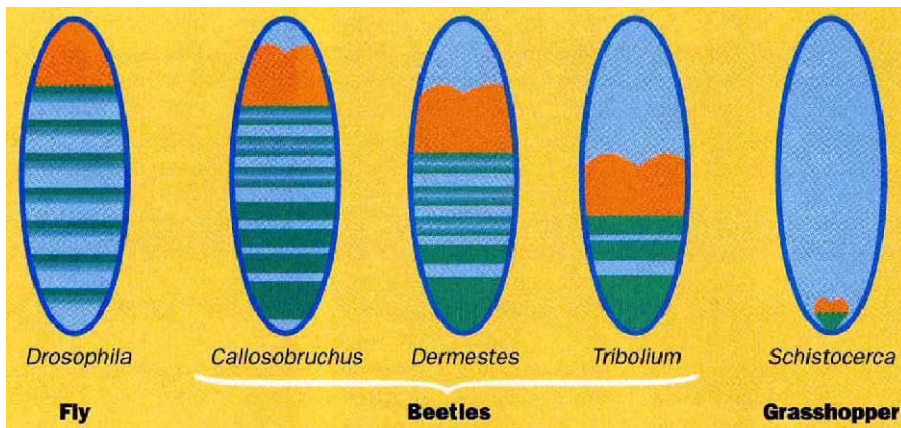
Is pairing the rule?

Michael Akam

THE earliest stages of development sometimes appear to be very different, even in closely related organisms. Do these differences imply the rapid evolution of new developmental mechanisms or does the apparent diversity mask fundamentally conserved machinery? Enough is now known about insect segmentation to address this question, and on page 429 of

paper in this issue⁴ (page 460) Nagy and Carroll show that its expression in the beetle *Tribolium* parallels almost exactly that in *Drosophila*, not only in the segmental stripes, but also in several other patterning processes.

The interesting comparisons concern how this pattern of opposing *wingless*⁺/*engrailed*⁺ stripes is set up. In *Drosophila*,



Patterns of *even-skipped* expression at the onset of gastrulation. In the long-germ *Drosophila*, the forming embryo fills virtually the whole egg. Pair-rule stripes of *even-skipped* protein already define the primordia for all segments. A similar pattern of *even-skipped* expression appears in the beetles, but at the corresponding morphological stage it is incomplete. Its extent correlates with the classification of the embryo as long- (*Callosobruchus*), intermediate- (*Dermestes*) or short- (*Tribolium*) germ band. In the extreme short-germ embryo of *Schistocerca*, *even-skipped* is expressed only in a posterior block: no pair-rule stripes ever appear. The anterior limit of the forming embryo is indicated by the head lobes (orange). In *Schistocerca*, these actually lie across the posterior pole of the egg.

this issue¹ Patel and colleagues conclude that the answer to both questions is 'yes'. Segmentation mechanisms that are fundamentally similar may seem to be very different if the relative timings of various processes are shifted, which accounts for the apparent diversity of development among the beetles. But a broader look at the different orders of insects suggests that more radical innovations accompany the transition from 'lower' to 'higher' insects.

The basis of insect segmentation is a juxtaposition of distinct cells to form a boundary that is self-perpetuating². On one side of the boundary, cells secrete the *wingless* protein. On the other, they express the *engrailed* gene, and emit a different signal, probably the *hedgehog* gene product (of recent fame). Only if these different cells are in proximity will they maintain their boundary states, yet they will not mix. Hence segmentation. This mechanism is probably ancient, and conserved in most if not all arthropods. Antibodies to *engrailed* protein reveal segmentally reiterated stripes in crustaceans as well as in diverse insects³. The role of *wingless* has been studied less widely among arthropods, but in another

a pre-pattern of double-segment periodicity is first established^{3,6}. This pattern comprises the striped distribution of several transcription factors, encoded by a set of genes dubbed the 'pair-rule' genes (so-called because mutations in the pair-rule genes delete alternate segments). The products of these genes provide the spatial cues that trigger the localized transcription of *wingless* and *engrailed*. Among the best studied are the genes *even-skipped*, *fushi-tarazu* and *hairy*.

The *Drosophila* pair-rule pattern forms early, in the syncytial blastoderm, and is complete at the time of gastrulation. However, in many other insects, the embryonic primordium is short at the time of gastrulation, and most segments appear to form later. Whether or not a double-segment pre-pattern arises in these 'short-germ' insects has been the source of some controversy. In a short-germ beetle, *Tribolium*, *hairy* transcripts appear in stripes prior to segmentation⁷, but in the grasshopper *Schistocerca*, *even-skipped* expression shows no trace of pair-rule patterning⁸.

Patel *et al.*¹ have examined this question by focusing on the beetles, where different

members of the same order have been classed as long-germ (like *Drosophila*), short-germ or intermediate. Using a monoclonal antibody to monitor *even-skipped* expression, they find that a pair-rule pre-pattern arises in each of these classes, although it presents itself rather differently in the beetles as compared with *Drosophila*. Instead of all pair-rule stripes appearing simultaneously throughout the embryo, as they do in *Drosophila*, *even-skipped* stripes in beetles appear sequentially, from front to back. Nonetheless, at least in the odd-numbered segments, the registration between double- and single-segment patterns is the same in beetles as in *Drosophila*: the activation of the *engrailed* gene follows precisely the anterior margin of each *even-skipped* stripe. (There is, however, a subtle difference in the way that *engrailed* stripes arise in the even-numbered segments, showing that beetle and fly segmentation have evolved some molecular differences.)

What then of the apparent differences between different beetles? Patel *et al.* conclude that the short-/long-germ classification reflects how far the sequential patterning of *even-skipped* stripes has progressed at the time of gastrulation (see figure) — but that does not imply that there are fundamental differences in the patterns of gene expression underlying segmentation. All three beetles show exactly the same resolution of double-segment *even-skipped* stripes into pairs of *engrailed* stripes.

Flies and beetles belong to a group of 'advanced' orders that represents a single, relatively late radiation within the Class Insecta⁹. The grasshopper *Schistocerca* is the only insect species outside this group that has been studied with molecular probes. Here, *even-skipped* expression tells a different and more enigmatic story⁸. The gene is expressed in the posterior part of the *Schistocerca* germ band, but its transcription never resolves into a repetitive striped pattern. *Even-skipped* protein has faded from any given cell before *engrailed* transcription is detectable. It is hard to reconcile this with a mechanism anything like that operating in *Drosophila*. The *even-skipped* gene lies at the heart of the *Drosophila* pair-rule system¹⁰. Its modular promoter interprets

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the positional cues provided by the local gradients of the gap-gene products in the syncytial blastoderm to generate a transcriptional output that embodies the first spatial periodicity in the embryo. Separate elements in the promoter refine this pattern by sensing the distributions of other products of pair-rule genes (*hairy*, *runt*) that act in parallel with *even-skipped*. Finally, its patterned output — the distribution of *even-skipped* protein — is used to regulate *engrailed*, *wingless* and probably other segmentation genes. The absence of spatial modulation leaves us with few clues to hint at what *even-skipped* is doing in the *Schistocerca* germ band.

It remains possible that a double-segment pre-pattern is generated in some different way in *Schistocerca*. There are tantalizing hints that double-segment periodicities underlie segmentation in several other groups of arthropods¹¹. But, at present, it seems most likely that pair-rule patterning, as we know it in *Drosophila*, is an innovation that has been invented stepwise during the diversification of the insects. □

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GAMMA-RAY ASTRONOMY

Bursts of controversy

Bradley E. Schaefer

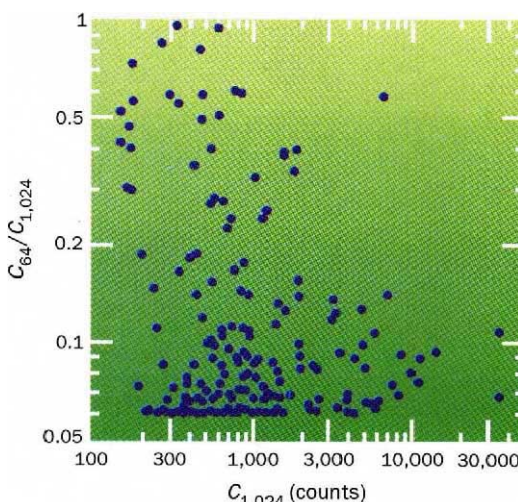
LAST December, one issue of the *Monthly Notices of the Royal Astronomical Society* carried four articles on gamma-ray bursts (GRBs), two of which claim important discoveries^{1,2} and two of which contradict these discoveries^{3,4}. The discovery papers state that GRBs recur every several months and originate in our Galaxy. If true, this would be a great step forward.

Gamma-ray bursts are short-duration flashes of gamma radiation that flare from random directions and at unpredictable times. The cause of bursts remains frustratingly unknown, with about 100 separate models to be found in the literature. In the past few years, proposed explanations have ranged from comets in the Sun's Oort cloud to jets from active galactic nuclei. The uncertainty in the distance to GRBs is 13 orders of magnitude, and this is a measure of our understanding.

The launch of the BATSE detectors on the Compton Gamma Ray Observatory has provided a wealth of new information, including the isotropy and inhomogeneity of bursters⁵. Don Lamb and colleagues have analysed BATSE catalogue data and from these derive three statistical correlations (refs 1, 2, 6; and manuscript in preparation). First, they identify two distinct morphological classes of bursts: type I bursts are smooth and long; type II bursts variable and short. Second, they conclude that GRBs repeat with a time-scale of months¹. Third, a selected subset of type I bursts are, they say, concentrated towards the galactic plane and centre².

These claims have important implications for the nature of bursters. If repeaters exist, this rules out the many extragalactic models (some currently popular) where the burst event destroys the entire system. Also, a concentration towards the galactic plane requires an origin in the Milky Way. This last conclusion is what many in the GRB community would like

to believe, because, before the BATSE results, the favoured model involved magnetized neutron stars in our Galaxy. Nevertheless, despite this bias, the community has reacted with considerable scepticism.



This plot of the variability of individual gamma-ray bursts ($C_{64}/C_{1,024}$) as a function of brightness ($C_{1,024}$) was used to divide the bursts into two classes, with the division set at $C_{64}/C_{1,024} = 0.16$. The scarcity of points in the upper right quadrant is one of the main reasons for postulating the existence of two distinct morphological classes of GRBs. The catch is that any plot of x versus y/x (or $C_{1,024}$ versus $C_{64}/C_{1,024}$) will tend to have a dearth of points in the upper right because of the intrinsic correlation of the plotted quantities. (After ref. 6.)

The first conclusion (that two morphological classes can be distinguished by variability) is based on an apparent lack of bright, variable bursts⁶. Here, brightness is defined as the BATSE peak count rate over a 1,024-millisecond time bin ($C_{1,024}$) and variability is defined as the ratio of peak rates over 64 and 1,024 milliseconds ($C_{64}/C_{1,024}$). Unfortunately,

things are not quite as simple as they might appear. The brightness is strongly correlated to variability by definition, so a lack of bright, variable bursts is expected regardless of intrinsic burst properties (see figure). In support of the classification is a correlation between the variability and duration (D. Q. Lamb and C. Graziani, manuscript in preparation), in that variable bursts are short in duration and the smooth bursts are long in duration. Again, though, there is the danger that this correlation could actually be a product of the definitions, in that short-duration bursts will be variable regardless of the shape of their light curve and the division by variability is largely a division by duration⁷.

Quashnock and Lamb's conclusion¹ that bursts are closely clustered on the sky, so they must recur, comes from a statistical analysis that finds an excess of nearest neighbours for angular separations of about 5°. For bursts with positions of moderate accuracy (known to within 9°), they find that this excess has a significance of 0.99975. Narayan and Piran, writing in the same issue³, re-analyse the data without making a cut on accuracy, and find that the significance is reduced to

0.9985. Things get rather worse when they use a two-point correlation analysis. This does not confirm the nearest-neighbour analysis, as it yields a significance of 0.948. Restricting the analysis to bursts whose positions are known to better than 4° reduces the significance to 0.425. This could be due to a bias against faint repeaters with uncertain positions, although then the excess clustering would be expected to have typical angular separations much greater than 5°. In fact, the two-point correlation function is near zero for separations greater than 4°, in violation of the repeater hypothesis, which demands a gaussian-shaped excess as a function of separation (D. Hartmann, personal communication). A potential problem arises because of the comparable excess of antipodal bursts, a result that suggests both clusterings are fluctuations^{3,8}. The question is only likely to be settled when we have larger numbers of bursts to go on. As yet, a subsequent and larger set of BATSE bursts shows

no clustering for any separation⁹, although this may be due to the use of unrefined positions.

What of the suggestion that type I bursters are concentrated towards the plane and centre of our Galaxy? This is based on the selection of bursts with intermediate peak count rates and with low variability, for which the concentra-

tion towards both the centre and plane are significant at the 2.5σ level². But this would invalidate the probability calculation used above¹ (D. Hartmann, personal communication), because a concentration towards the galactic plane will produce systematically closer neighbours than expected for an isotropic distribution. Unfortunately, the use of peak count rates² depends sensitively on the orientation of BATSE. According to Rutledge and Lewin⁴, in the final paper of the set of four, if these detector effects are removed then the significances of the concentrations towards the galactic plane and centre are substantially reduced (1.8σ and 2.4σ). Later BATSE data (using roughly triple the number of bursts) with the same selections show a slight concentration towards the galactic anticentre and no concentration towards the galactic plane¹⁰.

So it looks like the biggest enigma in modern astrophysics remains unsolved, and new techniques will be needed. A confident counterpart identification will probably settle the question, and very rapid response by ground-based instruments may soon be possible with positions from the HETE spacecraft or the BATSE Coordinate Distribution Network. Or the distance scale could be measured from the photoelectric absorption in the soft X-ray band. These and other experiments offer hope that we in the GRB community will soon know whether we are planetary astronomers or cosmologists. □

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■ At last month's meeting of the American Astronomical Society (Washington DC, 11–15 January) a cosmological origin for gamma-ray bursts seemed to be coming to the fore. According to Jay Norris (NASA Goddard Space Flight Center), two systematic trends can be seen in the 700 or so bursts detected by the Compton Gamma Ray Observatory so far: the fainter bursts are systematically longer and 'softer' than their brighter brethren. The results remain tentative, but both effects would be consistent with (although not require) a cosmological distance scale, with fainter, more distant bursts at higher redshifts. **L.M.**

Trinity of cation channels

Thomas J. Jentsch

ONE of the most satisfying moments in science is when different lines of investigation converge to yield a beautiful picture that opens up new perspectives. This happened last year when expression cloning^{1,2} of an epithelial sodium channel subunit revealed that the DNA encoding it was significantly similar in sequence to that of certain nematode genes, mutations in which lead to insensitivity to touch, neurodegeneration or both. Three reports

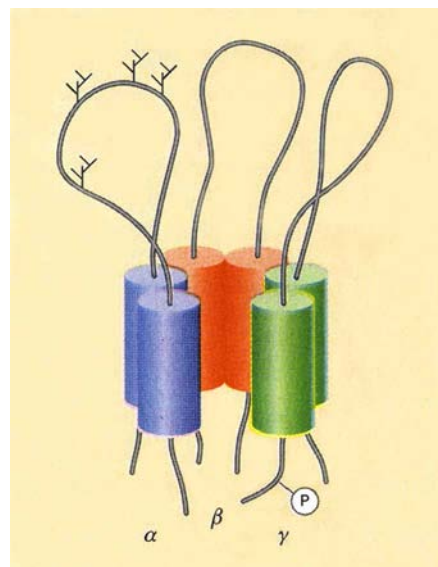


FIG. 1 Model of an epithelial sodium channel. A functional channel may contain more than one of each subunit, or other unknown subunits. Only the α -subunit is glycosylated, and the γ -subunit has an intracellular consensus site for cAMP-dependent phosphorylation. Each subunit is shown as spanning the membrane twice, but Canessa *et al.*³ propose that additional β -sheets may participate in the formation of the pore.

on pages 463, 467 and 470 of this issue^{3–5} now suggest that at least three distinct subunits are used to build channel complexes in both mammals and the nematode *Caenorhabditis elegans*. Further, the new work provides insights into the relationship between subunit structure and function, and demonstrates a remarkable degree of functional conservation between vertebrates and invertebrates.

Sodium channels allow selective diffusion of sodium ions across the plasma membrane (concentration and potential gradients will allow only net influx). Voltage-dependent sodium channels mediate the rapidly inactivating inward currents underlying action potentials. In contrast to the primarily electrical function of the voltage-dependent channels, epithelial sodium channels mediate bulk flow of Na^+ (and water, which follows

osmotically) across cell layers. These channels are not strongly voltage-dependent and do not show rapid inactivation, but are subject to complex regulation (for example by aldosterone and vasopressin) on a longer timescale. They are blocked by submicromolar concentrations of amiloride; the effect of amiloride on sodium reabsorption in the distal nephron of the kidney is the basis for its clinical use as a diuretic and in the treatment of hypertension. From physiological evidence, it seems that there may be a family of related channels⁶.

Whereas the first voltage-gated sodium channel was cloned by Numa's group almost ten years ago⁷, the biochemical identification of epithelial Na^+ channels has proved difficult^{8,9}. The approach that finally proved to be successful was expression cloning in *Xenopus* oocytes^{1,2}. Expression of the single cloned complementary DNA induced currents with many of the expected properties. However, currents were smaller than those observed with expression from tissue polyA⁺ RNA. This pointed to the involvement of other subunits, and led Rossier's group to search for them (they termed their first clone product the α -subunit of the rat epithelial sodium channel, α -rENaC)¹. In a new triumph for expression cloning, Canessa, Rossier and colleagues have now cloned³ two additional homologous subunits by functionally screening pools of cDNA clones expressed together with the α -subunit. Co-expression of all three subunits (α , β and γ) leads to much larger currents, and nearly all the known properties of the native channel are reproduced.

Epithelial sodium channel subunits define a new class of ion channel which is structurally unrelated to the neuronal sodium channel. They are predicted to have two hydrophobic transmembrane domains, separated by a large extracellular segment (see Fig. 1). As expected, sequence similarity is highest in the transmembrane domains, which presumably participate in forming the ion-selective pore.

It came as a pleasant surprise that the epithelial sodium channel is related by sequence to the *C. elegans* degenerins, which are encoded by the *mec-4* and *deg-1* genes among others, and which were identified in a genetic screen for touch insensitivity¹⁰. What Huang and Chalfie⁴ have now done is to identify a new member of this family (*mec-10*); and by studying *mec* mutations and their genetic interactions in transgenic nematodes, these investigators, and Hong and Driscoll⁵, have come up with an intriguing view of

degenerin activity.

In analogy to the epithelial sodium channel, MEC-4, MEC-6 and MEC-10 proteins are proposed to form heteromultimeric ion channels in *C. elegans* touch receptors (see Fig. 2a). These channels, which are necessary for touch sensitivity, may be directly mechanosensitive but could also function in signal amplification. Dominant mutations at equivalent positions in either the *mec-4* or the *mec-10* genes result in unregulated channel activity, which eventually leads to swelling and degeneration (lysis) of the touch cells. These gain-of-function phenotypes can be suppressed by certain loss-of-function alleles of the same genes (*mec-4* or *mec-10*), implying that several copies of MEC-4 (or MEC-10) insert into the same complex. The products of the recessive alleles thereby abolish the unregulated channel activity, without, however, restoring touch sensitivity. Surprisingly, however, most loss-of-function *mec-10* alleles enhance *mec-10*-induced degeneration, maybe indicating that these products are now unable to associate.

Although *mec-10*-induced degeneration requires both *mec-4* and *mec-6* activity, dominant *mec-4* alleles do not need *mec-10* activity to cause degeneration. This suggests that MEC-4 also participates in the formation of a different channel (lacking MEC-10) which is not involved in touch perception (Fig. 2b). The work of Canessa *et al.*³ also points to variable subunit stoichiometries; here, subunit messenger RNAs show differential tissue distribution and α -subunits are sufficient to form functional channels, albeit inefficiently. Degeneration of different nematode neurons by *deg-1* mutations also depends on *mec-6* activity¹¹, indicating that those channels contain at least DEG-1 and MEC-6 protein (Fig. 2c). Additionally, rare dominant mutations in the *unc-105* gene cause severe muscle contraction¹², which can be suppressed by mutations in other genes. *unc-105* is now known to belong to the degenerin gene family (R. H. Waterston, personal communication), suggesting the existence of another, related nematode channel and a similar pathological mechanism.

The new results^{4,5} also show that several loss-of-function mutants in *mec-4* and *mec-10* affect highly conserved residues in their products' second transmembrane domain. Many of these residues are predicted to lie on one side of an α -helix, suggesting that they line the pore. The region that is most fully conserved between different members of this protein family is the second transmembrane domain, and the beautiful domain-swap experiments of Hong and Driscoll⁵ show that these regions of DEG-1 and α -rENaC can specifically replace the equivalent segment of MEC-4 without destroying its function.

This picture is attractive, but validating it requires more evidence. It needs to be determined by molecular cloning whether *mec-6* is a degenerin gene. Further, the stoichiometry of the channel complex (in both nematodes and mammals) will have to be elucidated. In this case, as for other multimeric channels, detailed functional analysis¹³ of dominant negative mutants may be useful. Finally, there is a pressing need for an electrophysiological analysis of *mec* gene products; for instance, a

cloning of pressure-sensitive channels of baroreceptors or vascular smooth muscle could provide novel candidate genes for hypertension, and may lead to new drug-targeting strategies. Another potential area of interest is cystic fibrosis. Most symptoms of this disease result from the loss of the intrinsic chloride channel activity of the cystic fibrosis transmembrane conductance regulator (CFTR). However, sodium reabsorption is increased in lung epithelia of cystic fibrosis patients,

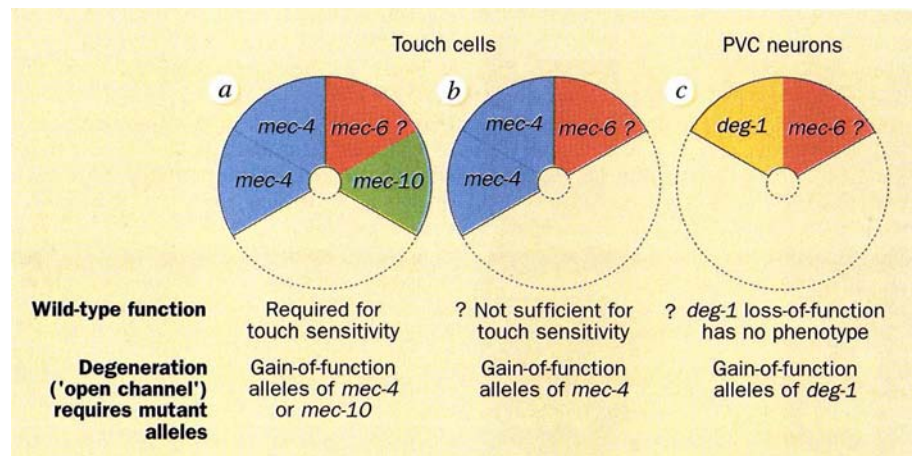


FIG. 2 Possible explanation of degenerin gene-family function in *C. elegans*. In analogy to the sodium channel, degenerins are proposed to form multimeric ion channels (in touch receptors, a, b or in other neurons, c); however, direct evidence for this is lacking. Each subunit is assumed to have a similar topology to that shown in Fig. 1. (The inclusion of *mec-6* in this scheme is purely hypothetical, as its membership of this gene family has not yet been established.) The fact that loss-of-function *mec-4* alleles suppress degeneration caused by gain-of-function alleles of the same gene suggests that at least two such subunits are present in a channel complex. This may also apply for *mec-10* (not shown). Other subunits may also be involved. Degeneration of cells is caused by dominant mutations, which are hypothesized to lead to nonphysiological channel opening. Degeneration of posterior ventral cord (PVC) interneurons caused by *deg-1* leads to a different form of touch sensitivity, because these cells relay signals from posterior touch cells¹¹.

demonstration that residues in the second transmembrane domain indeed line the pore will require evidence of changes in ion selectivity in mutants.

What are the broader implications of this research? First, a large channel family may now be amenable to molecular analysis, and it may include mechanosensitive channels that have some biophysical and pharmacological properties in common with epithelial sodium channels. Such channels are involved in hearing, in sensing of stretch and pressure, and possibly in regulation of cell volume. How they sense mechanical force is unclear, although the cytoskeleton has often been implicated in the process. Interestingly, touch receptors of *C. elegans* specifically express bundles of large-diameter microtubules, and mutations in *mec-7* (which encodes a β -tubulin) lead to a loss of these bundles and of touch-sensitivity¹⁴.

Second, there are the clinical aspects. In a rare hereditary form of hypertension (Liddle's syndrome), the increased sodium reabsorption in the distal nephron could be due to defects in the epithelial sodium channel or in its regulation. The

and it will be important to see whether or how CFTR regulates the epithelial sodium channels. It would also not be surprising if some forms of human neurodegenerative disorders are caused by defects of related channels. □

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An unstable superconveyor

Wallace S. Broecker

THE glacial portion of the record kept in Greenland ice is riddled with large and abrupt shifts in oxygen isotope ratio and dust content. These climatic changes have been attributed to switches in the mode of operation of the Atlantic Ocean's thermohaline circulation¹. Ocean dynamicists were quick to take up the challenge to simulate this phenomenon, and indeed have succeeded. Many of their models exhibit multiple states of operation. By releasing excess fresh water to the surface of their model's northern Atlantic, they can produce sudden switches in circulation from one mode to another, sometimes followed after a thousand years or so by an abrupt recovery to the initial state. This success has led to a general agreement that while it was surrounded by ice sheets the Atlantic Ocean's thermohaline circulation was vulnerable to meltwater-induced mode switches, and that these switches were responsible for the large temperature changes in the lands surrounding the northern Atlantic. By contrast, during the Holocene era, when large ice sheets were absent, the Atlantic's circulation was well behaved.

Then, with the publication last July² of an oxygen isotope record from the Summit site in Greenland, this simple concept of a noisy glacial Atlantic and a quiet interglacial Atlantic came under challenge. The oxygen isotope ratios and dust contents for the last interglacial period

displayed abrupt shifts akin to those that characterize the glacial portion of the record. Once again, the dynamicists have risen to the challenge. On page 447 of this issue, Weaver and Hughes³ present model results which suggest that the stronger hydrological cycle expected to accompany the warmer than Holocene climate of the last interglacial could have induced instabilities in the Atlantic's circulation.

Their simple two-basin model ocean exhibits three stable states. In all three, two Atlantic circulation cells are operative: a shallow conveyor driven by deep-water formation at its northern end, and a deep conveyor in the opposite sense driven by deep-water formation in the Southern Ocean. The states differ in the relative strengths of these circulation cells. The conveyor's water flux varies from 1.5 sverdrups in its weak mode to about 10 sverdrups in its median mode and about 25 sverdrups in its strong mode. As the heat transport to the atmosphere over the northern Atlantic varies in proportion to the conveyor's strength, Weaver and Hughes take these three modes to be analogues for the three temperature regimes that characterized the Greenland record during the last interglacial.

They also succeed in inducing cycles involving millennium-long intervals during which the strong conveyor mode is operative. These intervals terminate with sharp transitions to the weak mode fol-

lowed immediately by a more gradual shift to a millennium-long stint in the medium mode. The cycle ends with an abrupt shift back to the strong conveyor. The authors drive this cycle by introducing large stochastic changes to the strength of the model's hydrological cycle (that is, in the freshwater flux to high latitudes).

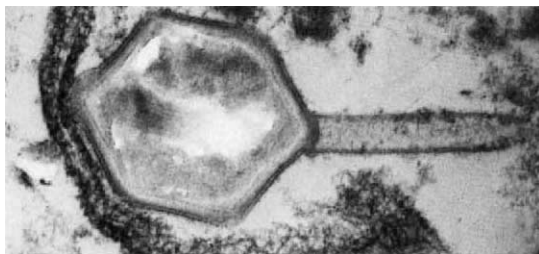
But two questions haunt this exercise. The first has to do with the reliability of the Greenland ice-core record for the Eemian time (~115–135 kyr ago). Late last year, published comparisons of the records for the two Summit ice cores cast serious doubt on the existence of the Eemian temperature fluctuations. Although the records for the upper 90 per cent of these two 3-km-long cores (drilled 30 km apart) are identical down to the smallest details, the lowest 10 per cent are not^{4,5}. They are so different that one or both must contain missing or repeated sections. Indeed, tilting, drag folding and thrusting of annual layers demonstrate that deformation has occurred in the lower several hundred metres of the ice column. Although the presence of ice with significantly higher ¹⁸O/¹⁶O ratios in both cores leaves no doubt that ice from a several-degree-warmer Eemian period is present, the dramatic shifts to cold separating these intervals of warmth could well be of tectonic origin; ice representing quite different climates may have been shuffled together by thrust faulting.

As were Weaver and Hughes, I was initially captivated by these abrupt Eemian shifts. But now, having studied the papers comparing the two records, having listened to and discussed the additional evidence presented at recent meetings in Berkeley and San Francisco, and having pondered why these large shifts in climate have never been recognized in the northern Atlantic marine or European pollen records, I find myself a Doubting Thomas. Only when matching records for the Eemian period have been obtained from a pair of Greenland ice cores will I be confident of the existence (or non-existence) of large and abrupt climate changes during times of interglaciation. But a decade is likely to pass before further such cores can be obtained and analysed.

The second question has to do with the suitability of the ocean models used to generate changes in the mode of thermohaline circulation. Anyone who has pondered their architecture is aware that the results depend heavily on the assumptions made with regard to the surface boundary conditions. The strengths of the conveyor and anticonveyor circulation cells that jockey for dominance in the models'

A particular mystery

THE nature of the object pictured here is not known. It comes from a vacuole of the phaeodarian radiolarian *Porospathis*, a marine protozoan. At first sight it looks like a virus. But if it is, it would be a Goliath of the sort, for its 'head' is well over 300 nm long, some



five times that of well-characterized marine viruses. So what else could it be? At present its status remains a mystery, as indicated by its loose designation as a large virus-like particle (LVLP). Such particles were recognized only a few years ago, and the latest report on their distribution has been compiled by Marcia M. Gowing (*Mar. Ecol. Prog. Ser.* 101, 33–43; 1993). Gowing has looked in particular at their occurrence in phaeodarians — so-called because of their possession of a phaeodium, the collective name for the food and waste vacuoles which are unique to the group and which provide a record of their food intake. Not only did LVLPs crop up in samples taken in various areas and at various depths, but Gowing estimates that a single phaeodarian could carry a cargo of over 5,000 of them. She thinks the phaeodarians acquire the particles during feeding, pointing out that it is unlikely that they are infected as such (LVLPs were never found in the cytoplasm or nucleus). And on the question of alternative identities, she offers the possibilities that they might be stages in the life cycle of some organism or other, eukaryote organelles or gametes, spores, or hitherto undescribed microorganisms. Whatever they are, the oceans are shot through with them.

T. L.

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jockey for dominance in the models' Atlantic are dictated by the density contrast between surface waters in the two polar regions. Because of this, the modellers can easily alter the nature of the response to forcing. So as Weaver and Hughes state, their model should be viewed as "process-oriented rather than predictive".

On the positive side, it is clear from the ice-core results that the northern Atlantic basin was considerably warmer during the last interglacial. Weaver and Hughes suggest that the extra heat responsible for this warmth was released by a conveyor oper-

ating far more vigorously than usual. They also suggest that the stronger hydrological cycle brought about by this extra warmth carries the seeds of instability preventing the stronger mode from remaining operative for intervals longer than about a millennium. These are most interesting possibilities which merit serious attention, especially as a global effort is currently under way to provide our planet with extra warmth. □

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SYNTHETIC CHEMISTRY

Sodium springs a surprise

Edwin C. Constable

SPONTANEOUS self-assembly of molecules is every synthetic chemist's dream. If molecular reactants contain the appropriate complementary molecular recognition features for intermolecular bonding, then simply mixing them can result in the formation of elaborate supramolecules. In metallosupramolecular chemistry these recognition processes and the intermolecular bonding are associated with the

interactions between metal ions and ligand donor atoms. Transition metal centres, with their strong and highly directional bonding, have been used for most such self-assembling systems. But on page 441 of this issue¹ Bell and Jousselin demonstrate that, given the right ligands, even group I metal ions such as sodium may control the assembly of double-helical supramolecules.

Oligopyridine ligands such as **1** are predisposed to form helicates on interaction with transition metal ions^{2,3} — predisposed as opposed to preorganized, because they must change their conformation before coordination can occur. With group I metal ions, the bonding is non-directional and inherently weaker, so ligands of type **1** will not readily form helicates. Bell and Jousselin therefore designed preorganized ligands such as **2**, in which the additional $-\text{CH}_2\text{CH}_2-$ bridging groups constrain the pyridine rings to a conformation in which they all lie on the same side, with all of the nitrogen donor atoms endocyclic. In the case of ligand **2**, the terminal benzylidene substituent 'slides' over the other end of the molecule to generate a helical structure.

When sodium salts are added to solutions of **2**, two different types of helical complex are formed. The first complex, **3**, contains a single sodium ion in the centre of a ligand array in which two molecules of **2** have

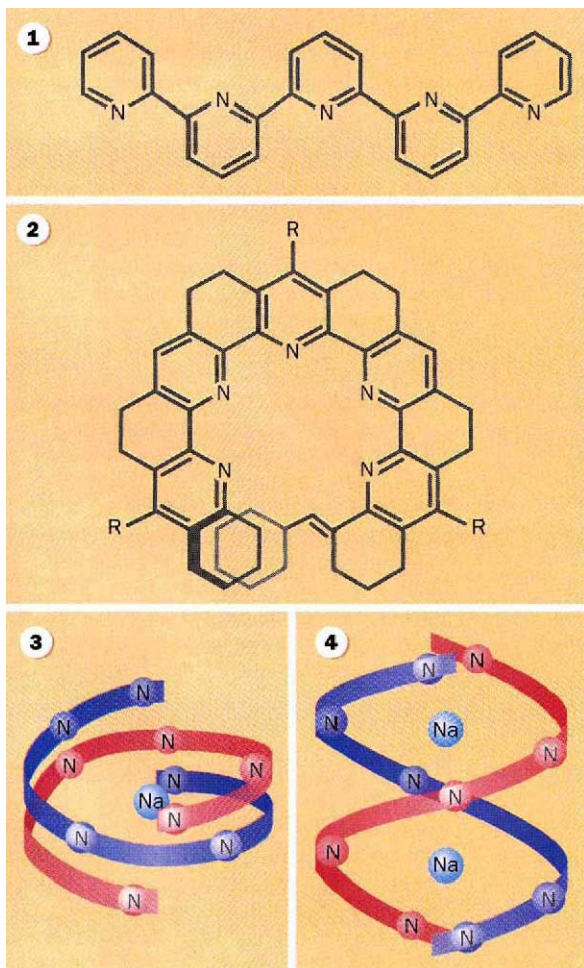
been twisted together to form a double-helical arrangement. In the second complex, **4**, the same double-helical motif is present, but it now supports two sodium ions. The double-helical array may be thought of as two intertwined springs which can be expanded or contracted, with resultant changes in pitch to accommodate different metallic cores.

Bell and Jousselin go on to demonstrate that, in solution, the two enantiomeric forms of the dinuclear complex **4** do not readily interconvert, although the parent preorganized ligand rapidly inverts on the proton NMR spectroscopic timescale. Their observations are the first detailed description I have seen of a double-helical supramolecule formed by the interaction of ligands with group I metal ions, although there is a tantalizing report of a "1:1" complex of **1** with lithium thiocyanate⁴. This compound may well be a double-helical 2:2 species.

To a generation brought up with crown ethers, the formation of group I complexes with pyridine nitrogen donors rather than ether oxygen donors may seem strange. But it is now well established that open-chain or macrocyclic ligands related to **1** and **2** form remarkably stable lithium complexes^{5,6}. The torand (cyclic ligand) related to **2** can be pictured by replacing the two overlapping rings shown in **2** by a single linking ring. Bell and colleagues have shown that the reaction of this torand with aqueous lithium salts results in the assembly of a solid-state supramolecular species in which the torand ligands are threaded about $\text{Li}-\text{OH}-\text{Li}$ chains⁷. These preorganized ligands offer great potential for the assembly of novel materials.

Cram and co-workers⁸ last year described another helical group I complex with nitrogen donors. There, the highly preorganized helically chiral ligand **5** (see next page) possessed a pseudo-tetrahedral cavity in which four endocyclic nitrogen donors are presented to a metal ion. Helical 1:1 complexes are formed between **5** and sodium or copper(I) cations.

It is more usual to find stable complexes of group I metal ions associated with cyclic or encapsulating polyether ligands, and such donor sets have been widely



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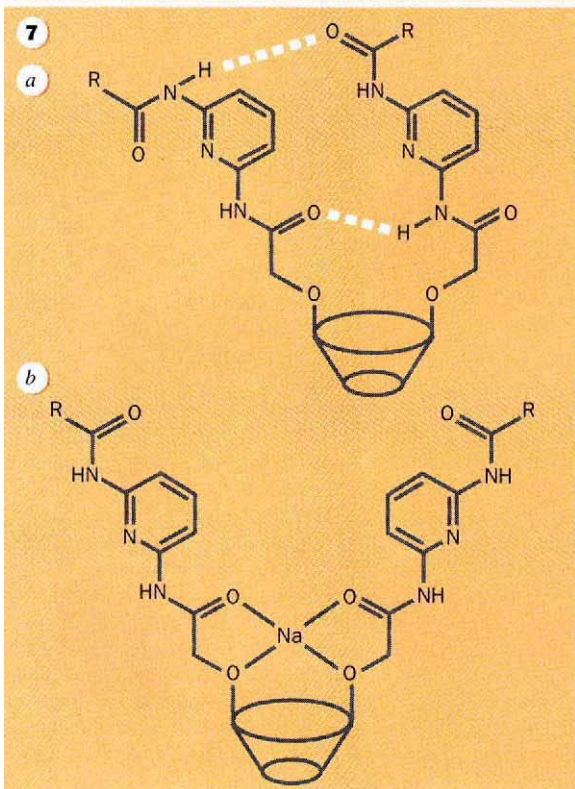
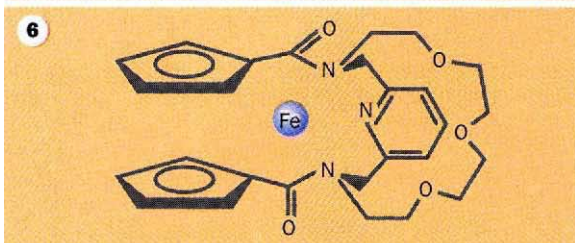
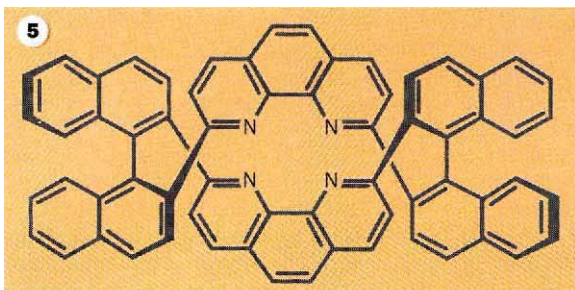
Warning twinges

LAST week Daedalus suggested that the brain is sensitive to radiation; that radioactive decay within it releases small doses of neurotransmitter at random. Oddly enough, some of the brain's neurotransmitters, such as serotonin and bradykinin, act in the body as pain transmitters. They are released from inflamed or damaged tissue, and trigger the nerves of pain.

Clearly, says Daedalus, creative evolution is working on a new pain sense to warn us against dangerous levels of radiation. To help it along, he is devising a radiation-sensitizing drug. Many enzymes are reversibly activated by changes of temperature or pH; DREADCO's biochemists are devising one that is activated by radiation. They are modifying the enzyme which controls the synthesis of bradykinin so that it is normally inactive, but can be flipped into its active form by the ionic track of a high-energy particle. It may then generate thousands of molecules of bradykinin, enough to launch a pulse into a pain nerve, before it flops back into its inactive form to await the next particle. (Rhodopsin in the eye is briefly flipped by visible photons in much the same way.) As one particle may create thousands of ion pairs, each of which may induce the enzymatic formation of thousands of bradykinin molecules, even a single particle might cause a brief but noticeable twinge of pain. When the new enzyme has been perfected, DREADCO will market it as 'Radiosense', the pill that lets you feel radiation.

Radiosense will usher in a new era of radiation safety. Its dosage will be chosen so that harmless or background radiation has no effect, minor warning levels are uncomfortable, while serious intensities become intolerable. It will be a wonderful liberator for physicists, X-ray technicians and nuclear-power workers. In effect, they will become their own Geiger counters: they will automatically sense every nuance of their radiation environment. Instead of obeying cumbersome and often misdirected safety rules, they will feel any problem that arises, and will be able to track it down immediately. The pain of a serious leak will force them to escape or don protective clothing, whose effectiveness or otherwise will be obvious at once. Safety will enforce itself: only a determined masochist could suffer radiation injury. Radiosense should also sell widely to ordinary citizens who are not sure how frightened they should be of X-rays, radon, reactors or even computer screens. The product will either reassure them, or give them solid grounds for complaint.

David Jones



incorporated into the components of supramolecular systems. A particularly exciting application is seen in the interactions of polypeptides modified by terminal 15-crown-5 groups with group I metal ions⁹. Sodium ions fit snugly into the cavity of 15-crown-5 to give 1:1 complexes, whereas the larger potassium cation forms 2:1 complexes in which the metal ion is sandwiched between two crown ether ligands. In the case of the modified peptide, interaction with potassium salts gives a bundle in which two α -helical rods are linked head-to-head. Molecules of this type will allow metal-directed bundle assembly processes to be probed, and have an obvious relevance to the changes in helix-helix association state that occur in biological ion channels.

not involved in intramolecular hydrogen bonding, and are available for intermolecular interactions. The hydrogen-bonding pattern (two donors and one acceptor) is complementary to that of isalloxazine, which binds only to the 'open' form **7b**. The result is that the system acts as a sodium-ion-activated detector for isalloxazines and related molecules.

Overall, and despite our preconceptions, the 'innocent' metal ions of group I and group II are increasingly playing important roles in the assembly of functional metallosupramolecular systems. □

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In another interesting development, Hall *et al.*¹⁰ have designed a molecular receptor containing a redox-active ferrocene group and a macrocycle with both pyridine nitrogen and polyether oxygen donor atoms (**6**). The ferrocene group acts as an electrochemical probe, and preorganizes the macrocycle for the binding of metal ions. When **6** interacts with calcium salts, the result is the assembly of a sandwiched 2:1 complex, in which the ferrocenyl groups are brought close together by the coordination of the macrocycle to the metal ion.

Finally, we may consider a functionalized calixarene reported by Murukami and Shinkai¹¹. Calixarenes are cyclic, bucket-shaped molecules which have been widely used as host molecules in supramolecular chemistry. In this case, Murukami and Shinkai are using the calixarene as a spacer for two pyridinediamide groups, which are attached to the 'rim' of the bucket. These two pendant groups are strongly hydrogen-bonded to each other to give the 'closed' ligand **7a**. Interaction of **7a** with sodium salts results in a conformational change to generate a binding site for the metal ion. The coordination of the sodium to two oxygen donors of each pendant chain results in a complex of the 'open' form of the ligand, **7b**. In this form, the three nitrogen atoms of each side chain are

Mismatch repair and cancer

SIR — Last March, a *Science* editorial hailed the discovery of a 'new cancer gene', linked to the hereditary nonpolyposis colorectal carcinoma (HNPCC) and localized to the human chromosome 2p16-15. The phenotype of the HNPCC cells appeared to be the instability of microsatellite DNA and other short repeats, and the accumulation of a large number of mutations scattered over the entire genome¹⁻³. This looked like a typical hallmark of a mutator gene at work and it did not take long before a link was made between the colorectal carcinoma phenotype and that of *Saccharomyces cerevisiae* mutants, *pms1*, *mlh1* and *msh2*, which are all deficient in mismatch correction⁴.

Further light was cast on the subject by Kolodner and colleagues⁵, who described the localization of the human *msh2* homologue, *hMSH2*, to chromosome 2p22-21 and demonstrated that this gene does indeed encode a mutator function, as overexpression of *hMSH2* in *Escherichia coli* gave rise to 10-fold higher mutation rates. Two weeks later, Leach and 36 coauthors⁶ followed up with a convincing study, reporting the localization of the *hMSH2* gene to chromosome 2p16. These authors also identified mutations, both germline and somatic, in the DNA of HNPCC patients. Both sets of authors concluded that *hMSH2* is the HNPCC gene and suggested that the protein encoded by *hMSH2* is probably involved in DNA mismatch repair. Modrich and colleagues⁷ substantiated these claims by showing that HNPCC-derived cell line H6 cannot repair mismatches *in vitro* nor *in vivo*. We can provide independent confirmation that *hMSH2* encodes a mismatch-binding protein.

In 1988, we reported the identification of a human (HeLa) cell protein, GTBP,

which could bind with high affinity to oligonucleotide duplexes containing G/T mispairs⁸. This protein which also recognizes mispairs other than G/T when purified⁹ appeared to possess no enzymatic 'repair' activity on mismatch-containing DNA duplexes *in vitro*. But GTBP does have ATPase and helicase activities, which is consistent with the properties of MutS, a mismatch-binding protein of *E. coli*¹⁰. This was a very interesting finding, as Crouse and co-workers¹¹ reported the identification of a gene, *Rep-3*, divergent from the mouse *Dhfr* locus, which contained an open reading frame very similar to the bacterial *mutS* and which also appeared to have a human counterpart, *DUG* (ref. 12). This serendipitous discovery rang numerous bells in the world of mismatch repair and, thanks to PCR, led to the rapid identification of a family of *mutS*-homologues, or *msh*, genes in yeast, mouse and human genomes (see ref. 13 and references therein).

Proteolytic digestion of the purified 100,000 GTBP polypeptide with trypsin, followed by sequencing of the generated fragments, yielded ten peptides: FR27-ECVLPGETAGDMGK; FR28-LTSLNEEYTK; FR30-GGILITER; FR39-MN FESFVK; FR40-IIQEFLSK; FR43-END WYLAYK; FR45-GDFYTAHGEDAL LAA; FR48-LLSAQFGYYF; FR52-ET LQLESAAEVGF; and FR60-ALELEE FQYIGESQGY. Using degenerate oligonucleotides based on the sequence derived from peptide FR60 to probe a lambda-ZAP HeLa complementary DNA library (Stratagene), we isolated a partial cDNA clone which contains approximately 1,000 nucleotides of the C-terminal part of the GTBP open reading frame. The DNA sequence of this partial clone matches that of *hMSH2* (refs 5, 6).

In addition, a consensus ATPase site and all the ten peptides above could be located in the translated *hMSH2* sequence, as shown in the figure. We therefore conclude that, based on the evidence presented above, *hMSH2* encodes GTBP.

As already stated, this latter protein appears to be a functional homologue of the *E. coli* mismatch-binding protein MutS, which plays a key role in the initiation of methyl-directed mismatch repair in this organism. Given that the principal roles of MutS are those of the recognition and binding of base-base mismatches¹⁰ and small loops¹⁴ as well as that of recruitment into the 'repairoosome' of the other participating proteins MutL, MutH and others, and given that the absence or malfunction of the MutS protein reduces the fidelity of replication of this organism by three orders of magnitude¹⁵, it is easy to understand the mutator phenotype of HNPCC cells where the *hMSH2* gene is non-functional.

The *hMSH2* story is clearly just the tip of an iceberg. One need only look at the complexity of the *E. coli* MutHLS methyl-directed mismatch repair system¹⁶ to realize that a phenotype similar to HNPCC could be due to any one of a number of mutations in the large number of genes involved in mismatch correction. All that remains now is to identify them.

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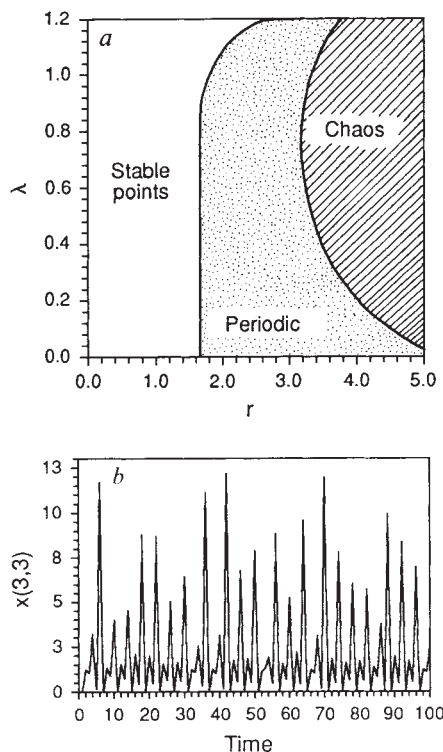
Amino-acid sequence of *hMSH2*, reproduced from ref. 6. GTBP peptides are boxed. Only a single deviation from the published sequence was found, an Asp to Leu mutation in peptide FR-48. The ATPase consensus sequence is underlined. (Note that the postulated *hMSH2* open reading frame published in reference 5 is missing the N-terminal 25 amino acid residues. This is due to a difference in the cDNA sequence, whereby the true ATG initiation codon appears as ATC and methionine 26 was thus taken as the translation start site).

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Ecological chaos

SIR — In his important contribution, Stone¹ analyses one-dimensional maps that exhibit period-doubling reversals using an additive constant immigration term (λ). Taking the Ricker's equation as a reference model, he shows that for increasing λ , reversal takes place and chaos becomes more difficult to observe. As a consequence, he concludes that chaotic dynamics in ecology may result from using simple models than from the real world.

We wish to raise some questions. One is how the constant 'immigration' term can be large enough to dominate density-dependence. Another involves the explicit introduction of space in ecological models²⁻⁶. Spatial degrees of freedom are able to generate complex dynamics together with ordered patterns²⁻⁴. If dispersal between patches is introduced, chaos is common for almost all parameter combinations, both for single species⁴ and for two-species models involving Lotka-Volterra³ or host-parasitoid² models. We have shown⁴ that the critical boundaries separating stable states from oscillations and chaos in the parameter space are strongly shifted even when very small lattices and diffusions are used.



a, State space for the spatial counterpart of Stone's model. We use a coupled map lattice^{3,4}. Lattice size, 6×6 ; diffusion coefficient coupling nearest neighbours, $D=0.05$. There is a well-defined region characterized by chaotic dynamics. Compare with Fig. 2 of Stone's paper¹. **b**, Local temporal series for the coupled map version of Stone's model for $\lambda=1$ and $r=5$.

We have now studied a 6×6 lattice model with diffusion coefficient D between the nearest patches. The results obtained for $D=0.05$ are summarized in the figure (a). As can be seen, even in such a small spatial domain, chaos emerges for enough high growth rates (r), even for very high λ values. With $D=0.1$, most of the previous periodic points become chaotic. The figure (b) shows an example of local chaotic oscillations obtained for $\lambda=1$ and $r=5$, whereas Stone reported only periodic orbits. As a consequence of diffusion, space starts to dominate the dynamics.

Concerning the stability properties, global populations in presence of spatial chaos have been shown to be nearly constant (stable)³⁻⁶. The origin of this stability is that spatial chaos leads to short correlation lengths and so patches tend to be independent. As a consequence, the global extinction probability is very small^{3,5}. Stone's results will encourage those working on chaos to carry the discussion further, and to examine the robustness of chaotic phenomena in ecology.

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STONE REPLIES — The reversal of period doubling cascades is a fundamental, if not inevitable, phenomenon common to many non-linear dynamical systems and strikingly apparent in even the simplest of difference equations¹⁻³. Although I commented and possibly over-emphasized the observation that period-doubling reversals may suppress chaos in simple models³, Solé and Bascompte imply that the phenomenon is absent in more complex systems. After criticizing an allegedly large immigration parameter in one of my examples — the Ricker map (the parameter was in fact a negligible perturbation) — Solé and Bascompte escalate the effects of this ecological process by allowing populations to migrate between a set of patches as well. But, unlike the Ricker map and more complex non-linear equations⁴, in their spatially extended model chaos occurs over a significant region of parameter space (but only at very high and possibly unrealistic intrinsic growth rate levels).

Are these findings in conflict with my general thesis? A preliminary examination of the spatial model of Solé and

Bascompte uncovers the existence of a complicated structure of period doubling and halving bifurcations, particularly in the parameter regime they classify as "periodic". This seems to reinforce rather than to contradict my basic argument. As it happens, there is a simple explanation as to why the Ricker model tends to suppress chaos more than the patch model, and it appears to have little connection with factors that concern spatial effects. My analysis of the Ricker model³ rested on a key working assumption advanced by McCallum⁵: given that in the real world there are numerous subpopulations that rely on refuges for protection or an influx of individuals by immigration, these organisms must have a 'floor' (λ) below which the population level never falls. It is this floor which enhances the initiation of a period-doubling since, in the terminology of my article, it forms a 'plateau' in the equation's associated return map³. However, for Solé and Bascompte's patch model, spatial diffusion enables populations to fall far below the set floor (λ) (see their figure b in which the population falls to zero when $\lambda=1$), and without preventative measures may even create absurd 'negative' populations. These effects destroy the 'plateau' in the model's return map, and thereby inhibit period-doubling reversals.

To test this possibility further, I incorporated McCallum's suggestion of a population floor in Solé and Bascompte's spatial model. The results are similar in character to that of the Ricker map — chaos being markedly suppressed together with the appearance of period-doubling reversals. Thus, the findings of Solé and Bascompte appear to differ not so much because of the novel spatial dimension incorporated into their model, but more likely because they violate a key working assumption.

My focus on the Ricker map was deliberate, for it provides a dramatic demonstration of the reversal of a period doubling cascade. This intrinsic phenomenon escaped the attention of a generation of mathematical biologists, who have been studying the dynamics of the Ricker map "under the microscope" for well over 20 years. In a similar way, period-doubling reversals are understood to be widespread in more general nonlinear systems. Should readers have any lingering doubts concerning this possibility, they will find further convincing evidence in the recent and intriguing studies of Dawson et al.^{1,2}.

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CO₂ and glacial cycles

SIR — Although global ice volume variations of near-100,000-year period are clearly the dominant mode of glacial variability in the late Pleistocene¹, there is still no widely accepted explanation for this phenomenon. It is recognized, for example, that while Earth-orbital (Milankovitch) radiative cycles are the likely explanation of the near-20,000- and 40,000-year period ice variations, they are certainly not sufficient to explain the much larger 100,000-year variations (though arguments have been made that they may be necessary). In one model, Milankovitch forcing acts only to set the phase chronology of the 100,000-year variation, but its existence and large amplitude is a consequence of a free non-linear oscillator driven by an internal instability of the carbon dioxide–ice volume–deep ocean circulation system^{2,3}. A fundamental prediction of this model is that the 100,000-year ice volume cycle must be out of phase with atmospheric CO₂, higher CO₂ values being associated with decreasing ice volume, and vice versa.

To test this prediction we can invoke two widely accepted, independent measures of CO₂ and ice volume: the Vostok core-trapped air measurements^{4,5}; and the SPECMAP $\delta^{18}\text{O}$ estimate⁶, respectively. Although questions arise concerning the absolute chronologies of these two measures, it is of interest to examine their implications taking them at face value as the best estimates currently available.

Some support for these estimates is provided by recent studies with regard to CO₂ (ref. 7) and to ice volume⁸.

In the figure we show the phase-plane trajectory of these two variables. If CO₂ and ice volume were in-phase, the trajectories would overlap, forming a straight line and indicating a diagnostic relation between the two. But in fact the trajectories tend to form two main 100,000-year period loops with a clockwise flow, signifying a phase lag between the two variables such that high CO₂ is associated with decreasing ice volume and lower CO₂ with increasing ice volume, as predicted. Smaller loops are also present, particularly during the time of lower CO₂, which have shorter periods corresponding to the Milankovitch obliquity and precessional cycles. These smaller loops can be either clockwise or counterclockwise, indicating randomness in the sign of the phase lag between CO₂ and ice mass on these shorter timescales. Because little direct orbital forcing exists at the 100,000-year period, and the thermal effect of a 100 p.p.m. change in CO₂ concentration is as large as that due to the Milankovitch radiative changes at 65 °N that presumably drive the near-20,000- and 40,000-year cycles^{9,10}, it is implied that CO₂ can provide the internal forcing necessary to drive the main near 100,000-year cycles.

From another viewpoint, the 'hole' in the 100,000-year period loop represents a region of phase space from which the climate system tends to be driven, indicative of a region where an unstable equilibrium might be located. On the other hand, points along the main trajectory, particularly where their density is great, are indicative of more stable regions of the

climate system.

It will be of great interest to extend the trajectory shown in the figure backwards in time as further Vostok CO₂ measurements projected to extend to 500,000 years before present⁵ become available, and to repeat this mode of analysis for other measures of ice volume⁸ or other revised estimates of the ice volume chronology that might turn out to be more accurate than SPECMAP.

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Mikhail Verbitsky

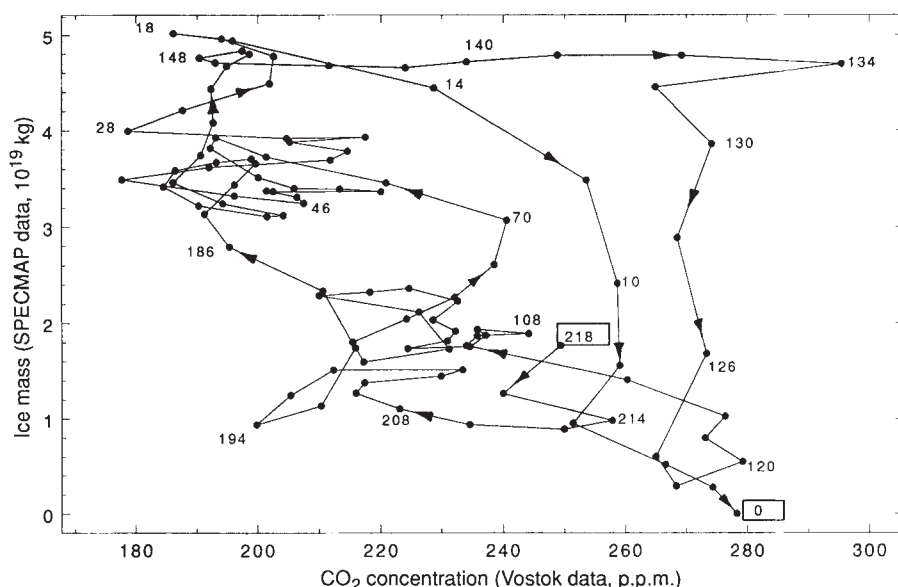
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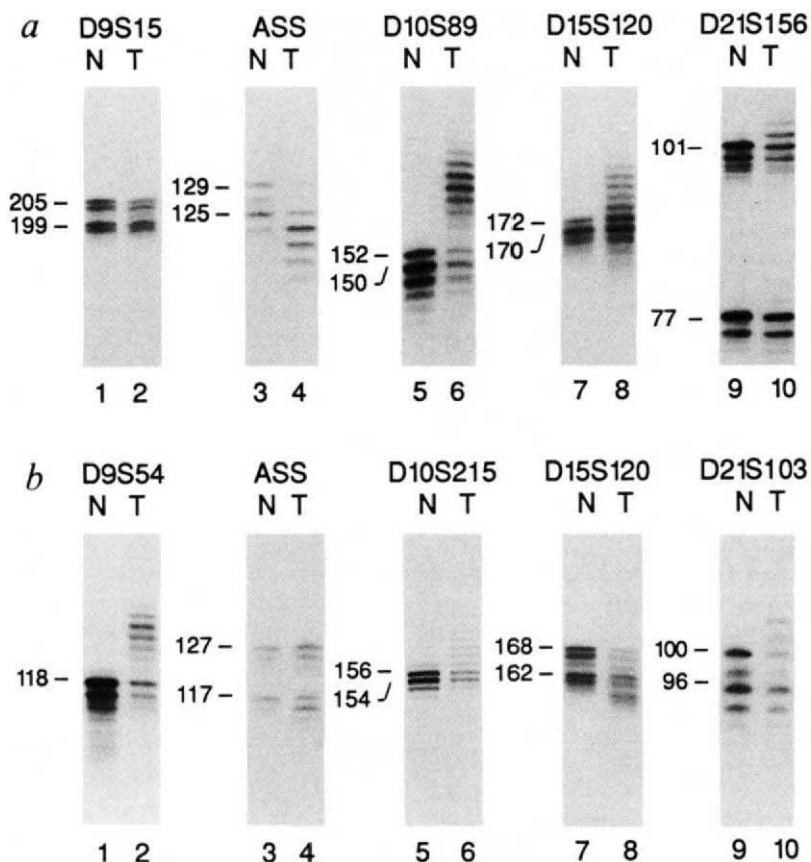
Urothelial carcinogenesis

SIR — Transitional cell carcinoma is a major genitourinary neoplasm arising from urothelial cells lining the bladder, ureter and renal pelvis. The frequent deletion of chromosome 9 in superficial carcinomas of this type has suggested that the condition begins by the functional inactivation of a putative tumour-suppressor gene. In attempting to localize this gene, we have applied the polymerase chain reaction (PCR)-based analysis of polymorphic simple sequence repeats or microsatellites to define regions of allelic loss on chromosome 9 in transitional cell carcinomas (ref. 1). Serendipitously, the use of these genetic markers has revealed another mechanism of urothelial carcinogenesis.

From a panel of 37 bladder, 3 ureter and 1 renal pelvis transitional cell carcinomas, we observed variations in the electrophoretic mobility of (CA)_n·(GT)_n microsatellite alleles in DNAs from a superficial ureter lesion (case 36), and an invasive ureter lesion (case 39), relative to DNAs from autologous peripheral blood lymphocytes. In case 36, we found mutant alleles at 16 (73%) of 22 loci on 4 of 5 chromosomes tested. The DNA from case 36, for example, was unaltered at D9S15, truncated at the argininosuccinate synthetase locus, and expanded at D10S89, D15S120 and D21S156 (*a* in the figure). In



Evolution of the global climate system in the phase plane of ice mass (10¹⁹ kg) as deduced from the SPECMAP reconstruction⁶ and carbon dioxide concentration as deduced from the Vostok ice core⁵. Numerals mark time ($\times 1,000$ years before present), beginning at $t=218,000$ years before present and ending at the present, $t=0$ (boxed). Each dot represents a 2,000-year time step. Arrows point in the direction of decreasing age towards the present.



Variation of microsatellite allele lengths in transitional cell carcinomas of the ureter. Shown are autoradiograms of PCR products from loci representative of those studied in cases 36 (a) and 39 (b). Genomic DNAs were isolated from surgically removed carcinoma specimens (T) and autologous normal peripheral blood lymphocytes (N). Microsatellites were assayed by PCR amplification of the DNAs with oligonucleotide primers flanking the repeating units, as described previously¹. Radiolabelled PCR products were separated on 7 M urea/6% polyacrylamide gels with an M13mp18 DNA sequence ladder as a size marker, and autoradiographed. Sizes of normal alleles are in base pairs. Other microsatellite loci studied: D9S168, D9S162, D9S171, D9S169, D9S161, D9S104, D9S43, D9S165, hexabrachion, D9S58, gelsolin, D9S67, D10S196 and D10S209. ASS, argininosuccinate synthetase.

case 39, mutations were equally frequent, being observed on all 5 chromosomes tested. For example, the DNA was expanded at D9S54, unaffected at the argininosuccinate synthetase locus, expanded at D10S215, truncated at D15S120, and expanded at D20S103 (b in the figure). Length variations were reproducible, occurred in 2-base-pair increments, and exhibited a 4:1 bias for sequence expansion. The frequency of microsatellite mutations in cases 36 and 39 was 700-fold higher than the spontaneous mutation rate². This phenomenon was not found to be a general characteristic of transitional cell carcinoma, as only 2 (5%) of 40 cases were affected. In the 38 unaffected cases, of 734 genotypes examined at 29 microsatellite loci, only 3 were mutated (0.4%).

An identical phenomenon was recently described in tumours from patients with hereditary non-polyposis colorectal cancer. Linkage studies of affected families have mapped susceptibility genes to

chromosome arms 2p and 3p (refs 3,4). Concomitant loss of heterozygosity was not observed in familial tumours, which is uncharacteristic of tumour-suppressor loci. Instead, widespread variations in microsatellite allele lengths were observed, suggesting that defects in the genes leads to errors in DNA replication or repair. Somatic mutations in the lengths of mono-, di- and trinucleotide microsatellites have also been observed in sporadic colon cancers⁵⁻⁷.

Of particular relevance to this study is the documentation of transitional cell carcinomas in some families with Lynch syndrome II, a variant form of non-polyposis colorectal cancer characterized by colonic and extracolonic cancers^{8,9}. The family history of patient 36 is suggestive of Lynch syndrome II. He developed transitional cell carcinoma at age 41, and his father had been diagnosed with bladder transitional cell and colorectal carcinomas. Given that Lynch syndrome II

individuals affected with transitional cell carcinoma have children who develop colorectal cancer⁹, and that genes for non-polyposis colorectal cancer have been associated with microsatellite allele length mutations in colonic tumours, a gene for non-polyposis colorectal cancer is probably responsible for the microsatellite mutations in the ureter transitional cell carcinoma of patient 36. Our molecular data are consistent with the possibility that transitional cell carcinoma of the ureter is among the spectrum of tumours associated with Lynch syndrome II. Patient 39 is a 75 year-old male diagnosed with both ureter and bladder transitional cell carcinoma, as well as adenocarcinomas of the lung and oesophagus. Although there is no available family history, and his adenocarcinomas have not been studied, the mechanism underlying the microsatellite mutations seen in his ureter carcinoma could explain his apparent susceptibility to cancer.

The microsatellite aberrations described in colon and transitional cell cancers are probably the secondary effects of a defective DNA replication or repair factor. Because there are approximately 100,000 (CA)_n·(GT)_n microsatellites in the human genome¹⁰, about 70,000 would be affected in cases 36 and 39. As (CA)_n·(GT)_n microsatellites are less prevalent than the (A)_n·(T)_n form¹⁰, a considerably higher number of microsatellite alterations could be expected. Most of these mutations would probably be inconsequential. However, length variations in trinucleotide repeats occurring within coding sequences of specific genes have been implicated in Fragile X syndrome, Huntington's disease and other human genetic diseases. Thus, it is conceivable that similar mutations in the appropriate genes could affect tumour development.

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Unknown agent

Eric Hall

Childhood Cancer and Nuclear Installations. Edited by Valerie Beral, Eve Roman and Martin Bobrow. *BMJ*: 1993. Pp. 453. £27.95, \$54.

THE gaudy but eye-catching cover of this book presumably shows the iridescent flow of waste from a symbolic nuclear installation. The title in shocking pink carries the unequivocal message *Childhood Cancer and Nuclear Installations*. It is dedicated to the late Martin Gardner whose work fanned the flames of controversy surrounding the "Seascale affair". It chronicles the events that began in 1983 with a programme by Yorkshire Television called *Windscale: The Nuclear Laundry*, which identified an apparent cluster of childhood leukaemias in the village of Seascale, which is on the coast close to the nuclear reprocessing plant at Windscale, subsequently renamed Sellafield. The television crew had gone to Sellafield itself to look into the possibility that the health of workers may have been affected, but their attention was drawn to what seemed to them an excessive number of leukaemias in local children. This report prompted a government inquiry, chaired by Sir Douglas Black, which concluded that there was indeed a higher incidence of leukaemia in Seascale. This spawned numerous investigations and discussions that have occupied epidemiologists and medical statisticians in Britain for a decade.

The book includes reprints of studies conducted in the United Kingdom, including Gardner's original papers, which are reproduced in full, as well as extracts from government reports and summaries of investigations conducted elsewhere in the world.

The introduction by the editors, Valerie Beral, Eve Roman and Martin Bobrow, is a useful and excellent summary of the unfolding story of the epidemiological evidence. Indeed, without it, it would be difficult for the uninitiated to make sense of the conflicting results of the studies that follow. If it has a weakness, it is the lack of an adequate summary of the objections raised to the Gardner hypothesis on genetic and radiobiological grounds, and the magnitude of the disparity involved.

With the appropriate boundary conditions (and they are a little bizarre) the Gardner papers showed an excess of 4 or 5 cases of leukaemia in Seascale, to the south of Sellafield, although there is no excess to the north, where most radiation workers live, or to the west in the path of the prevailing wind.

When Gardner's papers first appeared he interpreted the excess as an environmental problem — specifically the result

of radioactive emissions to the general population — resulting in the exposure of the children who subsequently developed cancer. It soon became apparent that the radiation doses to the children were much too low to account for the excess incidence. Later, the explanation of the excess was changed to an occupational problem, because the affected children had been fathered by workers at Sellafield who received substantial doses before

conception. The study was regarded as well conducted and statistically sound, but many scientists found its conclusions hard to accept, especially when follow-up studies in other countries failed to provide any evidence to confirm Gardner's conclusions.

It is interesting and pertinent that this book has appeared soon after the decision in the landmark case in the English High Court in October 1993 that ruled against the families of two of the affected children. They had claimed that the children's leukaemia and lymphoma had been caused by pre-conceptional irradiation of the fathers while working at Sellafield. It is significant that the defence in the case involved not only the epidemiology but also much cellular and molecular radiobiology, in the light of which it appears that the Gardner hypothesis is untenable.

For those who followed the evidence in

this lengthy court case, this book must seem somewhat unbalanced, with a surfeit of epidemiology but little science. To be fair, the book does include the papers that describe an alternative explanation, put forward by Leo Kinlen, and supported by Sir Richard Doll, to the effect that the leukaemias were due to an infective agent resulting from the influx of large numbers of people into previously remote areas of Cumbria. Leukaemia clusters similar to that noted at Seascale have been observed in sites proposed for a nuclear facility, but where the reactor was never activated, and also in remote areas developed for oil installations. This observation supports the idea that the Seascale excess, if it is not simply a statistical aberration, is due to some unidentified factor, but is not associated with radiation,

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Too close for comfort? — Houses near the Sellafield nuclear reactor.

The intended readership of this book is unclear. It cannot be meant for epidemiologists because there can be few who have escaped exposure to Gardner's papers. It cannot be for the mythical 'man-in-the-street' who (although perhaps attracted by the cover and title) would be lost in odds ratios and statistical significance. I hope it is not intended for general scientists (who are not specialists) because it is a limited selection of epidemiological papers with little basic science perspective. Who then is it for?

Prospective readers may be attracted by the eye-catching cover or by the striking title but after reading the contents they may feel, as did Macbeth, that "it is a tale full of sound and fury, signifying nothing". □

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Was Crookes a crook?

William Brock

The Sorcerer of Kings: The Case of Daniel Dunglas Home and William Crookes. By Gordon Stein. *Prometheus*: 1993. Pp. 140. \$22.95.

In 1861 William Crookes discovered thallium spectroscopically. For 10 years he patiently developed techniques to determine its atomic weight accurately. All weighings were conducted in a partial vacuum. While operating with his balance in an evacuated container, Crookes noted that the equilibrium of the balance was disturbed by slight differences in temperature between his thallium samples. Warmer bodies appeared lighter than colder ones. Because, in a vacuum, this could not be attributed to condensation or air movement, Crookes concluded that he had stumbled on a 'signpost' linking heat with gravitation.

In the same decade, spiritualism had swept across Europe from the United States and spread through all levels of Victorian society. Although Crookes came from a large family and many of his siblings had died in childhood, the death of his brother Philip at sea in 1867 affected him so deeply that Crookes began to seek assurance of Philip's immortality in the seance room. It was here that he met the Scottish-American medium, Daniel

Dunglas Home (1833–86), the model for Browning's Mr Sludge.

Although Home seems never to have indulged in physical mediumship, his amazing powers included levitation, elongation of the body, the ability to play an accordion without his fingers touching the keys and the depression of spring balances. Crookes and Home became firm friends, and when Crookes investigated Home between 1869 and 1870, he obviously connected Home's ability to exert a 'psychic force' (as Crookes termed it) with anti-gravitation and the effects of warmth in reducing the weights of bodies in his evacuation experiments with thallium. With Home as his experimental tool, might not Crookes be able to give a rational explanation of the psychic powers of mediums and cap this with an explanation of gravity?

The result was the radiometer. Initially developed to test mediums' mysterious powers, the instrument soon led Crookes to the electric light bulb, the world of cathode rays and dark spaces, the separation of rare-earth elements and to wonderful speculations about the evolution of the elements.

The creative connection between the investigation of mediumship and the origins of nuclear physics is not, however, the purpose of Gordon Stein's book which is, rather, a critical reexamination of Crookes's psychic investigations between 1869 and 1875. He concentrates his fullest attention on the reliability of Crookes's tests of Home, reopening in the process the case against Crookes's honesty first made by Trevor H. Hall in *The Spiritualists* (Duckworth, 1962) and by Eric J. Dingwall in *The Critics' Dilemma* (1966). Hall's inference that Crookes had a sexual affair in 1872 with the pretty, young physical medium, Florence Cook, was undermined by the brilliant detective work of R. G. Medhurst and K. M. Goldney in the *Proceedings of the Society for Psychical Research* in 1964. Nevertheless, Stein believes that Crookes was either too ashamed ever to admit that he had been duped by her or, alternatively, that he must have fraudulently conspired with Florence either for sexual favours or to support the claims of spiritualism.

More seriously, he argues that Crookes conspired to help Mrs Fay in 1875 when a galvanometer test 'proved' that she remained under restraint during her physical mediumship. Because both Cook and Fay were demonstrably tricksters, Stein is led to reexamine Crookes's investigation of Home. The latter's strange abilities are explained as known conjuring tricks as well as by Home's careful control of the conditions Crookes thought he was imposing on Home. Contrary to received opinion, Stein also shows that Home was exposed as a fraud on several occasions, besides being a skilled confidence tricks-

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Sir William Crookes portrayed by Spy in *Vanity Fair*, 1903.

ter. In that case, Crookes appears either gullible (the pseudo-Crookes, as W. B. Carpenter labelled him in *Nature*) or guilty of duplicity.

Although I do not doubt that Crookes's belief in spiritualism, or theosophy, affected his scientific judgment of mediums, Stein's conclusion (supported in a preface from the redoubtable James Randi) that Crookes was crooked is not fully supported by the surviving (admittedly fragmentary) evidence, much of which is cited in the book. Because Stein uses Dingwall's silly description of Home as 'sorcerer of kings', a more plausible explanation lies, perhaps, in the identity of a sorcerer's apprentice. The apprentice, however, was not Home's but Crookes's. An assistant who was ingenious enough to blow glass into beautiful radiometers and Crookes tubes and who was young enough to play poltergeist with his master and his guests might easily have been persuaded to 'assist' in the wonderful delusions Home, Cook and Fay constructed. It may be significant, therefore, that Crookes's most brilliant assistant left his employ in 1880, by which time Cook and Fay, if not Home, had been exposed as fakes, and Crookes had assumed his ortho-personality. Stein makes his case against Crookes and Home clearly and logically; but it is by no means the last word on one of the oddest affairs in the history of science. □

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Home's "amazing powers" included levitation and elongation of the body.

Observational cosmologist

Donald E. Osterbrock

Edwin Hubble, the Discoverer of the Big Bang Universe. By Alexander S. Sharov and Igor D. Novikov. *Cambridge University Press*: 1993. Pp. 187. £19.95, \$34.95.

EDWIN Hubble was one of the most important astronomers of all time. Copernicus and Galileo moved the centre of our Universe from the Earth to the Sun; Hubble moved it from somewhere in our Milky Way system to an arbitrary point in the vast, expanding universe of galaxies. He was born more than a century ago, and died in 1953. Yet, apart from memorial biographical articles written soon after his death, there were no biographies of him in print before this book.

Edwin Hubble, the Discoverer of the Big Bang Universe, written by two outstanding scientists of the former Soviet Union, originally appeared in Russian in 1989. Now, with only slight changes and additions, it has been published in an English translation. It is in many ways a good book. But a really satisfactory biography of Hubble remains to be written.

The authors are among the best representatives of Russian astronomy and astrophysics. Alexander S. Sharov is an outstanding observational research astronomer at the Sternberg Astronomical Institute. He has worked on our Galaxy and other, nearby galaxies, especially M 31 and M 33, which were the subjects of two of Hubble's most important early papers. Igor D. Novikov, now the director of the Theoretical Astrophysics Centre in Copenhagen, is a brilliant theoretical cosmologist.

The sections of the first part of the book, based on Hubble's published scientific papers, and on those of his predecessors who first saw dimly the redshift-apparent magnitude (or velocity-distance) relationship which was his revolutionary discovery, are very good. The authors found the published source material, understood it thoroughly and explain it very clearly. Similarly, the second part of the book, on the more recent observational and theoretical advances in the study of the Universe, is excellent. It is well written and informative. The main threads of the leading ideas in cosmology up to just a few years ago are very clearly explained. Fred Hoyle, Geoffrey Burbidge and Jayant Narlikar might complain

that the subtitle of the book, by describing Hubble as "the discoverer of the big bang", has pushed their ideas out of consideration, but it is hard to imagine a better treatment of mainline cosmology than the last 50 pages of this book.

Where the book fails, however, is in its treatment of its subject's life and of his human qualities. As the authors write in the preface, they could not do any research on the original sources for this side of Hubble's life. They had to depend on copies of some of the material in Hubble's personal papers in the Huntington Library in San Marino, which are themselves incomplete, on a tape-recorded interview with Allan Sandage, who worked with Hubble at the end of his life and knew him well, and on a letter from Hubble's younger sister, the late Helen Hubble Lane, who was 89 years old when she wrote it.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Hubble liked to embellish stories of his exploits.

Hubble himself, like many Americans but perhaps more than most, liked to act out roles and tell embellished stories of his exploits outside the field of science. After his death, his widow, Grace, selected and improved on these tales in a manuscript 'diary' and record of his life, which is in the Huntington Library. Richard C. Tolman, Hubble's close friend and collaborator, wrote a short memorial biography soon after Hubble's death which perpetuated some of these myths. Nicholas U. Mayall, Hubble's protégé and Grace's friend, did the same at greater length 15 years later. They are part of the canon of Hubble's life on which Sharov and Novikov necessarily depended.

In 1989, three US scientists and historians, who had dug into many sources for letters, news items, newspaper and magazine articles, high-school, university and

vital records, and First World War records and divisional histories, published an article exploding many of the myths about Hubble. Sharov and Novikov saw this article soon after the Russian edition of their book had been published but, as they themselves state, were able to make only slight and unsatisfactory changes to the English edition. Hence Hubble's personal life is not adequately described or interpreted in this book.

Its authors, quite understandably, do not know much about other US astronomers of Hubble's time. Thus they confuse Fred Wright, the geophysicist and an expert on the Moon and optical glass, with William H. Wright, the Lick Observatory spectroscopist. And they believe that the Barnard Medal of Columbia University, which Hubble received in 1935, commemorates the astronomer Edward E.

Barnard; actually it is named after Frederick A. P. Barnard, one-time president of the University of Mississippi and later of Columbia. Sharov and Novikov do not understand many of the nuances of American life, particularly the family pressure on Hubble to become a lawyer, and his own consuming but thwarted ambition to become the director of Palomar Observatory after the Second World War.

In the Russian edition, the names of Western scientists were naturally transliterated into Russian characters; for the English translation they were transliterated back using different protocol. Thus many well-known astronomers' names are misspelled or misstated. Similarly, several US cities are misplaced or have their names misspelled, and the National Academy of Sciences appears as the American Academy of Natural Sciences in one place in the book, but under its right name in another. The anonymous editor was clearly well schooled in English literature, for the quotations from Robert Louis Stevenson and Barbara Tuchman are correct, but astronomy was another matter.

Edwin Hubble is well worth reading for what it is: a scientific history based on Hubble's published research papers. But it is incomplete and even wrong as the story of his life, and of the thoughts and ideas behind those papers. Since 1989, more historical papers have treated other aspects of Hubble's career. His biography remains to be written. It will have to incorporate all these source materials, and probably others as well, still unknown or undiscovered. □

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Healthy antidote

Harold Morowitz

Uncommon Sense: The Heretical Nature of Science. By Alan Cromer. Oxford University Press: 1993. Pp. 240. \$23, £15.95 (hbk).

UNCOMMON Sense is an exposition of naive realism that is uncommonly terse in providing justification for the many assertions that are made. Thus Cromer attacks Thomas Kuhn's view of scientific revolution, the egocentric epistemology of the Judaeo-Christian tradition, the search for extraterrestrial intelligence and much of the current teaching of science. The author also expounds Piagetian psychology, chaos, hominid evolution, classical Greece and Israel, Egyptian and Babylonian mathematics, Arab science, Aristotle, mystical India, bureaucratic China, mediaeval Europe, capitalism and so on. In such a presentation, depth must of necessity fall victim to breadth.

Cromer's brevity is a pity, for many of his attacks on what we might call the Baconian idols of our time are probably called for and certainly deserve discus-

sion. His assertion that science is the unique product of Athenian rationality as it came to fruition in the past three or four hundred years of Western European culture is sufficiently politically incorrect to merit careful study, but care is not rendered. For example, Cromer asserts: "It was the institution of free debate more than anything else, I believe, that set Greece above all other nations." This of course ignores the tradition of debate recorded in the Talmud, the origins of which go back to the time of Ezra, who may have been a contemporary of Plato. This was the work of those he describes as egocentric in their ways of knowing.

His assertion that Kuhn's revolutionary approach is applicable to a developing science but not to mature sciences is intriguing, but how do we know when a science is mature? Cromer would probably regard astrophysics as mature, but, after black holes, quasars and dark matter, who would be certain we don't have a few more revolutions ahead of us? Again, the subject needs more careful exposition.

The five-page section headed "Aristotle" deals almost entirely with mechanics and celestial mechanics. This ignores the one-third of Aristotle's writings devoted to biology. Indeed, most sections of this

book devoted to the nature of science tend to avoid biology completely. In contrast, the section on hominid evolution uncritically assumes the certainty of some results from the very immature and argumentative science of palaeontology.

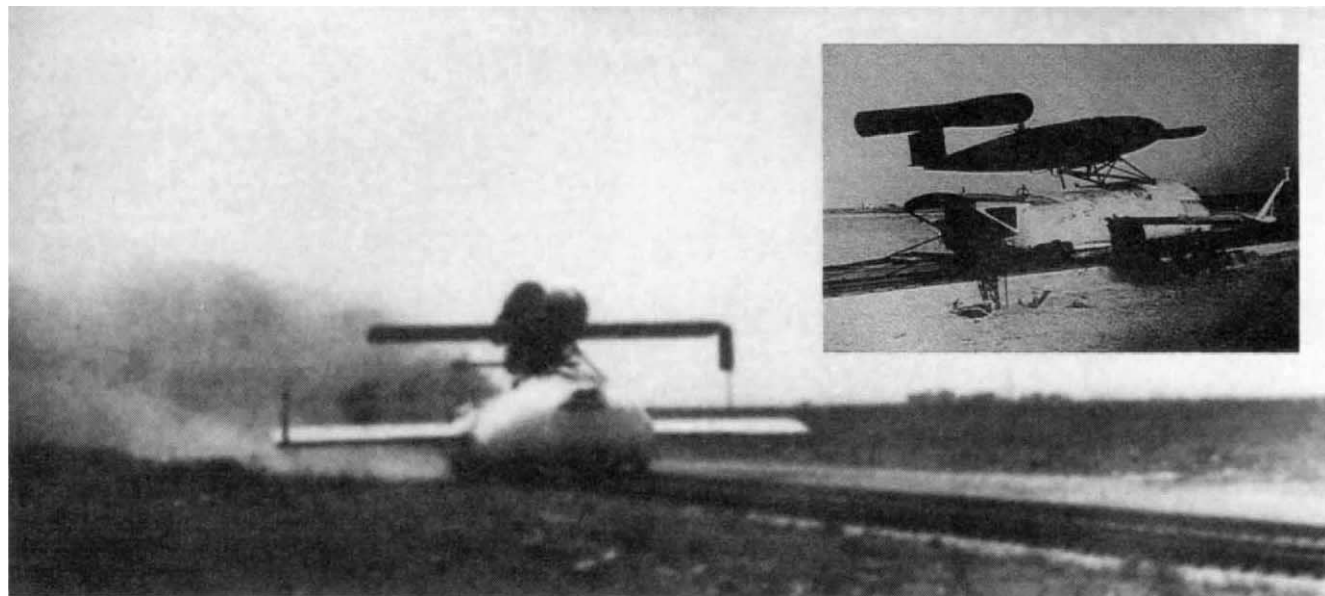
I suppose it is because I believe that Cromer is correct in some of his iconoclastic views that I find it disappointing that they are not pursued in a more scholarly fashion. In contrast, this book is a healthy antidote to all the deconstructing of the remarkable achievements of Western science that is going on in modern academic life. □

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New in paperback

The Ant and the Peacock by Helena Cronin. Cambridge University Press, £12.95, \$19.95. For a review see *Nature* **356**, 623 (1992).

The Creationists: the Evolution of Scientific Creationism by Ronald L. Numbers. University of California Press, \$15. For a review see *Nature* **360**, 637 (1992).



ON the 28 March 1952 at Istres, near Marseilles, this rocket-powered sled reached an incredible 328 kilometres per hour, shattering the previous record for rail vehicles of 196 kilometres per hour set in 1937 by the Bugatti "Présidentiel". The sled, known as the SE 1910, was a French secret military machine "far ahead of its time" that was to be a reusable launch vehicle for heavy ground-to-ground type missiles. Plans were also made to use the sled to launch piloted aircraft. It is shown here with a V-1 rocket, developed by the Germans in the Second World War, attached for the purpose of testing.

What made this sled unique was a combined acceleration and deceleration system, making use of rockets (powered by liquid hydrogen peroxide and calcium permanganate) for braking as well as propulsion. As well as the braking rockets (retro rockets) there

were two other braking systems: disk brakes damped by pressurized oil and a 'skid' system in which skids were lowered onto the track and four 'jaws', incorporating friction material, gripped the track. (Interestingly, in 1981 this system was suggested, though not used, as an emergency braking system for the French high-speed train, the TGV.)

There were 52 tests, all apparently without mishap, before the programme was halted in 1953. By this time, missile technology had advanced to allow heavy loads to be launched under their own power and smaller missiles could be launched from aircraft. There was no longer a need for such launching sleds.

From *History of Rocketry and Astronautics* edited by Lloyd H. Cornett Jr. American Astronautical Society History Series, Volume 15, \$60 (hbk), \$40 (pbk).

How B and T cells talk to each other

Edward A. Clark & Jeffrey A. Ledbetter

The B cells of the immune system, which secrete antibodies against foreign antigens, are fully specific and effective only after maturation in lymph nodes and other lymphoid tissues. Immunocompetent T cells play a crucial part in this process, but the molecular details of the way in which the two cell types interact have only recently become apparent.

MILLIONS of newly formed lymphocytes leave the bone marrow daily¹. Each of these cells either multiplies and differentiates or dies, depending on the nature, timing and location of interactions with other cells of the immune system^{2,3}. Specific recognition of foreign antigen by cell-surface immunoglobulin (Ig) induces B cells to proliferate and differentiate either into plasma cells, which produce soluble immunoglobulin to fight infection, or into memory B cells which can respond rapidly to subsequent encounters with the same antigen.

This process, however, requires help from activated T cells. Antigen-specific T-cell activation is dependent on interactions between T cells and specialized antigen-presenting cells, which can themselves be B cells. Once active, the T cells in turn promote B-cell activation, both by releasing T-cell-derived cytokines such as interleukin (IL) -2, -4 and -5, and by direct intercellular contact²⁻⁶. What follows is an account of some of the molecules responsible for the second part of this process.

Germinal centre formation

Resting B cells circulate in the blood, migrating across high endothelial venules to sites of trapped antigen in secondary lymphoid organs such as lymph nodes, the spleen, tonsils and Peyer's patches (Fig. 1a). B cells bearing immunoglobulin specific for trapped antigen enter the T-cell-rich paracortical regions below the outer layer, or cortex, of lymphoid tissues^{2,4,5}, where they capture and process the antigen⁷. Some T cells in this area may already have been activated by recognizing antigen fragments bound to the major histocompatibility complex (MHC) class II on antigen-presenting cells (Fig. 1b, c). Once activated, the cell-surface receptors of the T cell and the cytokines it releases induce antigen-bearing B cells to migrate into B-cell follicles; other types of antigen-presenting cells, such as the bone-marrow derived dendritic cell, may also fulfil this function.

In the follicle, B cells interact with follicular dendritic cells. Unlike dendritic cells, these large spiny cells are not derived from the bone marrow; they specialize in binding antibody-antigen immune complexes and in inducing B-cell proliferation and differentiation. This interaction leads to the formation of a finely structured germinal centre (Fig. 1c). Initially, rapid B-cell proliferation gives rise to a dark zone, relatively devoid of T cells (Fig. 1d). Maturing B cells then move into the light zone, which contains abundant follicular dendritic cells and some T cells. It is here, probably within the basal light zone, that the variable (V) region immunoglobulin genes of B cells undergo somatic mutation to increase their affinity for antigen⁸. This process occurs within days of antigenic stimulation^{8,9}, and only during germinal-centre development⁵. B cells with high-affinity receptors for antigen are subsequently 'selected', and switch from producing IgM to another immunoglobulin class. This step requires activated B cells, CD4-positive T cells and follicular dendritic cells (Fig. 1e). Eventually, germinal-centre B cells mature into either memory or plasma B cells, perhaps in response to signals in the apical light zone, which is rich in a subtype of follicular dendritic cell^{2,4}.

Signals that 'talk'

A number of the receptor-coreceptor pairs mediating the effects

of T cells on B-cell maturation are very well characterized (Fig. 2). In the paracortical region of lymphoid tissue (Fig. 1c), bone-marrow derived dendritic cells are very effective at presenting MHC class II-bound peptides to CD4⁺ T cells, as they express high levels of MHC class II and CD40, as well as key accessory molecules such as CD54, CD58 and CD80 (formerly termed B7 or BB1). Although the affinity of T-cell receptors for MHC class II-bound peptide is low¹⁰, the CD4 and CD2 coreceptors on T cells help signalling through the receptor after binding to MHC class II and CD58 (LFA-3), their respective ligands on the bone-marrow derived dendritic cells. Crosslinking of the T-cell receptor and its associated coreceptors in turn leads to the rapid activation of the CD11a/18 (LFA-1) complex¹¹, which can then bind to ligands such as CD54. The fact that bone-marrow derived dendritic cells express CD54 as well as CD58 and CD80 (Fig. 1b) may explain why naive T cells can be activated by these dendritic cells but not by resting B cells, which lack these receptors^{12,13}. Once B cells have been activated first by T cells (Fig. 1c) and later by follicular dendritic cells (Fig. 1e), they do express an array of surface receptors (Fig. 1e) and can present antigen to T cells effectively.

Cell-surface adhesion molecules, such as integrins (for example CD11a/18), selectins and CD58/59, are all broadly distributed, and are likely to affect the migration and adhesion of lymphocytes in general and to modulate lineage-specific signals. Other molecules are relatively restricted to antigen-presenting cells, T cells or B cells, and must therefore function in processes unique to lymphocyte maturation (Fig. 2). Two such molecules, CD40 and CD80, which are neither integrins nor selectins, bind coreceptors whose levels are affected by the state of the T cell expressing them (Fig. 2). These receptor-ligand pairs allow T and B cells to sense the state of their partner's activation.

CD40 and its ligand

CD40, a surface glycoprotein of 45–50K relative molecular mass, is related to the receptors for tumour necrosis factor (TNF)- α and is expressed on late pre-B cells in bone marrow, mature B cells and certain accessory cells, including bone-marrow derived dendritic cells and follicular dendritic cells (Fig. 1). Crosslinking CD40 promotes B-cell proliferation^{6,14}, prevents apoptosis of germinal-centre B cells⁴, and promotes immunoglobulin class switching¹⁵. The ligand for CD40, CD40L (also called gp39, 5c8 or TRAP), is a TNF family member expressed on activated but not resting T cells^{16,17}. When the CD40-CD40L interaction is blocked *in vitro* with soluble CD40 or monoclonal antibodies to CD40L (gp39), B cells cannot proliferate or produce immunoglobulin in response to T-cell signals (refs 16–19, and see ref. 20 for review), indicating that the interaction is required for signalling. Resting T cells that lack CD40L cannot help B cells.

Recently, the gene encoding CD40L has been shown to be defective in patients with hyper-IgM (HIM) syndrome²¹. Although these patients have IgM-producing B cells, they do not form germinal centres in response to foreign antigen. Their B cells are capable of switching from IgM to IgG or IgE production *in vitro* when exposed to IL-4 and monoclonal antibodies against CD40, but they do not switch immunoglobulin classes *in vivo*. T cells and CD40L-CD40 interactions are thus required

for both germinal-centre formation and immunoglobulin class switching, but not for at least some IgM responses.

As such interactions can theoretically take place either in T-cell zones (Fig. 1c) or in basal light zones (Fig. 1e), it is not yet clear where the CD40L-CD40 interactions necessary for germinal-centre formation or class switching take place. Interestingly, patients with HIM syndrome are also prone to infections with pathogens such as *Pneumocystis carinii*, prevalent in T-cell deficiencies such as AIDS²². Antibodies against CD40L block the development of both primary and secondary antibody responses²³ and of collagen-induced arthritis²⁴, confirming the key role of this interaction in B-cell regulation.

CD40 is expressed on follicular dendritic cells, bone-marrow derived dendritic cells, activated macrophages and thymic epithelial cells; CD40-CD40L interactions may thus regulate T-cell maturation via interactions with non-B cells. Crosslinking CD40 on some non-B cells has indeed been shown to augment cytokine production (see, for example, ref. 25).

The CD80 family and CD28/CTLA-4

Whereas the CD40L-CD40 interaction enables the B cell to respond to an activated T cell, the interaction between CD80 and CD28 allows peripheral T cells to respond to an activated B cell by dividing and producing cytokines required for T-cell differentiation. CD80 was originally defined as a B-cell activation marker^{26,27}, but it is now known to be one of a family of related molecules that share the same receptor and are found on B cells, activated macrophages and some bone-marrow derived dendritic cells^{28,29}. Of the ligands for the CD80 family, CD28

(ref. 30) is found on both resting and activated T cells, whereas the other, CTLA-4 (ref. 31), is found only on activated T cells. The binding of CD80 to CD28 on T cells previously stimulated through their antigen receptors increases IL-2 production and T-cell proliferation³².

The CD28 signal is discussed in detail elsewhere³³. Interference with this signal *in vitro* can block T-cell proliferation³⁴ and B-cell maturation induced by T-cell cytokines³⁵. A soluble form of CTLA-4 (CTLA4Ig) interferes both with CD80-dependent signalling to T cells *in vitro*³¹ and with primary antibody responses in mice, demonstrating the importance of CD80 in B-cell maturation³⁶. By contrast, weakly immunogenic tumours transfected with B7 complementary DNA induce a protective T-cell response^{37,38}, showing that CD80 expression can dictate whether or not cellular antigens are 'recognized' by the immune system.

A reciprocal dialogue

When and where might the CD28-CD80 interaction occur? CD80 expression is induced or stimulated after crosslinking MHC class II with monoclonal antibodies³⁴, during allogeneic T-cell interactions with bone-marrow derived dendritic cells³⁹, and during autoreactive T-cell interactions with B cells⁴⁰. Interactions with T cells which crosslink MHC class II may thus induce CD80 expression in B cells so that CD80 can, in turn, signal to the T cell via CD28. Induction of CD80 expression indeed requires the cytoplasmic tail of MHC class II⁴⁰.

CD80 expression may therefore coincide with CD40L expression on T cells responding to MHC class II-bound peptides (Fig.

FIG. 1 Journey of a naive antigen-specific B cell during the development of a primary T-cell-dependent response (after refs 2 and 4).

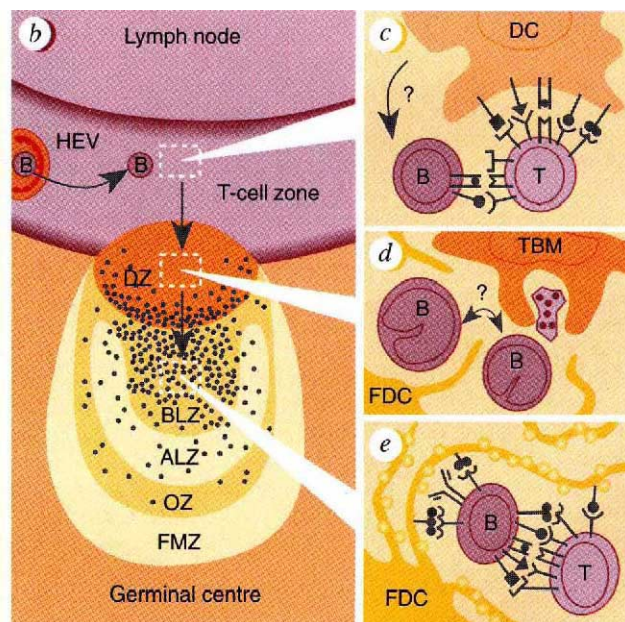
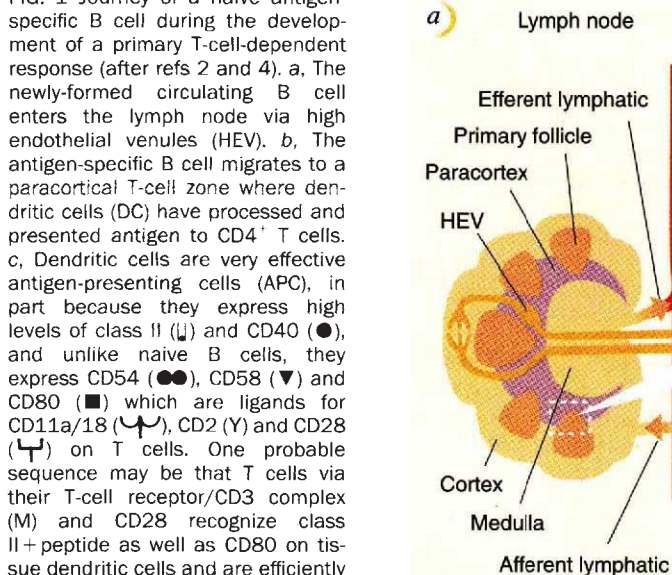


FIG. 1 Journey of a naive antigen-specific B cell during the development of a primary T-cell-dependent response (after refs 2 and 4). a, The newly-formed circulating B cell enters the lymph node via high endothelial venules (HEV). b, The antigen-specific B cell migrates to a paracortical T-cell zone where dendritic cells (DC) have processed and presented antigen to CD4⁺ T cells. c, Dendritic cells are very effective antigen-presenting cells (APC), in part because they express high levels of class II (□) and CD40 (●), and unlike naive B cells, they express CD54 (●●), CD58 (▼) and CD80 (■) which are ligands for CD11a/18 (↖), CD2 (Y) and CD28 (↗) on T cells. One probable sequence may be that T cells via their T-cell receptor/CD3 complex (M) and CD28 recognize class II+peptide as well as CD80 on tissue dendritic cells and are efficiently activated to express activated CD11a/18, CD40L (↖) and cytokines which in turn contribute to the activation of B cells. Unidentified signals induce a small number of activated B cells to migrate to a follicle where they begin to proliferate. d, Activated B cells become localized in the follicle, associate loosely with a network of follicular dendritic cells (FDC), and begin a rapid and oligoclonal proliferation followed by hyper-somatic mutation of B-cell immunoglobulin V-region genes, which leads to expansion to up to 10,000 B cells in 3 days; as a result, a dark zone (DZ) containing B centroblasts and tingible body macrophages (TBM) (d) and a light zone (LZ) containing a dense FDC network and centrocytes (derived from centroblasts) (e) are formed. d, Within the basal light zone (BLZ), some centrocytes with high-affinity IgM receptors (↖) are stimulated by specific antigen retained on CD54⁺ follicular dendritic cells and are selected to survive after crosslinking of surface IgM or CD40 (ref. 2) while other centrocytes not selected undergo apoptosis and fall back

in the dark zone where they are devoured by tingible body macrophages. The interaction between activated B cells, which express a full battery of cell-cell interaction molecules, and follicular dendritic cells may require CD49a (VLA-4) (↖) and CD106 (VCAM-1) (●●●) and CD11a/18 and CD54. Selected B cells may process and present antigen to CD4⁺ CD40L⁺ T cells, which can be found in the basal light zone⁴³; these T cells in turn may induce the B cells to undergo immunoglobulin class switching, to survive and to proliferate as memory B cells, processes involving CD40 crosslinking, IL-4, IL-10 and other cytokines. In the apical light zone (ALZ), B cells interact with CD23⁺ follicular dendritic cells and receive signals via IL-1, CD23 or other surface receptors, and thereby may be induced to become plasma cells or to become memory B cells. OZ, Outer zone; FMZ, follicular mantle zone.

3). Antibodies to MHC class II and CD3 indeed induce expression of CD80 and CD40L, respectively, with similar kinetics^{19,34}. Crosslinking CD28 on activated T cells also increases the expression of CD40L (S. Klaus *et al.*, manuscript in preparation), and crosslinking CD40 on B cells increases the expression of CD80 (ref. 41) and B70/B7-2 (ref. 28). This reciprocal dialogue may occur in peripheral lymphoid T-cell zones after T-cell activation, where CD40L⁺ T cells have been identified in both mice and humans^{42,43}, or in the basal light zones of germinal centres in humans, where CD40L⁺ T cells have also been found⁴³.

Controlling the dialogue

Interactions between T and B cells must be carefully regulated in order to prevent activation of self-reactive or bystander cells. The presence of CD40 and MHC class II on resting B cells, or the T-cell receptor/CD3 complex and CD28 on resting T cells, is insufficient to trigger mutual T-B activation^{3,12,13}. Indeed, rather than activating resting T cells, resting B cells tolerate them to specific antigens³. A reciprocal dialogue can ensue, however, if either the T or B cells are activated (Fig. 3). Activated T cells expressing CD40L induce resting B cells to express CD80 (ref. 41) and activated B cells expressing CD80 induce T cells to express CD40L (S. Klaus *et al.*, unpublished data). Signalling through CD40 on B cells or CD28 on T cells is most efficient after stimulation through their respective antigen receptors^{6,33}, ensuring that the interaction is specific.

Remarkably, the signals delivered by CD40 and CD28 have similar effects. Both promote cell proliferation stimulated by antigen receptor crosslinking^{6,14,33}. Moreover, crosslinking of CD28 prevents specific unresponsiveness or apoptosis that would otherwise occur on stimulation of the T-cell receptor^{44,45}. Similarly, signals delivered by CD40 on contact with T cells can block apoptosis of immature B cells induced by crosslinking their surface immunoglobulin receptors⁴⁶.

The CD40L-CD40 and CD28/CTLA-4-CD80 ligand pairs (Fig. 3) are clearly not the only means by which T cells and B cells interact. The CD11a/18-CD54 ligand pair (Fig. 2) is also likely to play a part, as: (1) an active form of CD11a/18 is rapidly induced on crosslinking the T-cell receptor complex¹¹; (2) antigen-specific T-cell activation rapidly induces a CD11a/18-CD54-dependent signal to the antigen-presenting B cell⁴⁷; and (3) crosslinking CD40 on B cells promotes allogeneic T-cell proliferation via CD11a/18-CD54-dependent interactions⁴⁸. Patients with leukocyte adhesion defect do not express CD11a/18 on their T or B cells. They make both IgM and IgG antibodies in response to specific antigen, but have depressed antibody titres, indicating that their production of memory B cells may be impaired⁴⁹. The CD11a/18 receptor may therefore contribute to T-cell-dependent B-cell maturation, but is not as essential as CD40L^{3,21}.

Surface IgM and associated adhesion molecules

Cell-surface immunoglobulin plays a critical role in several stages of B-cell maturation, including the pre-B-cell and immature B-cell stages in the bone marrow⁵⁰. Crosslinking of cell-surface IgM by antigen-bearing follicular dendritic cells also prevents B cells in germinal centres from undergoing apoptosis⁴. A number of surface molecules and protein kinases are associated with cell-surface IgM⁵⁰; how these molecules contribute to its signalling activity is unclear. Two B-cell-specific adhesion molecules, CD19 (ref. 51) and CD22 (ref. 52) (both members of the immunoglobulin supergene family), may associate with surface IgM and are rapidly phosphorylated on tyrosine residues after it becomes crosslinked^{53,54}. CD22 may be required for normal signalling, as intracellular calcium is not elevated after crosslinking surface immunoglobulin in CD22-negative B cells (see ref. 54 for review). CD22 also interacts with several ligands on B and T cells containing α 2,6-linked sialic acids⁵⁴, including the activation marker CD45RO, and is therefore likely to affect antigen-specific B-cell maturation.

CD19 is expressed on B cells throughout their maturation. Although its ligand has not yet been identified, it interacts with various molecules on other B cells, including the receptor for the C3d component of complement, CD21/CD2 (ref. 55), indicating that it may facilitate a complement-mediated signalling pathway. When crosslinked, CD19 induces calcium mobilization in both pre-B and mature B cells. After surface IgM becomes crosslinked, phosphorylated tyrosine residues in CD19 are capable of binding to the SH2 domains of proteins such as the tyrosine kinases c-abl and PI-3 kinase. CD19 indeed binds to the SH2 domains of PI-3 kinase after crosslinking of surface IgM^{53,56}. It may therefore bring key signal-transducing enzymes to the plasma membrane.

Discussion

The CD28-CD80 and CD40-CD40L receptor-ligand pairs are essential for the dialogue between B and T cells. Both CD28- and CD80-deficient mice have normal levels of B and T cells and produce immunoglobulins of all classes^{57,58}, indicating that the immune system has evolved alternatives to the CD28-CD80 pathway; these are now known to include CTLA-4 (ref. 31) and B70/B7-2 (refs 28, 29). By contrast, CD40-CD40L has no backup; individuals defective for CD40 (ref. 59) or CD40L²¹ do not form germinal centres or switch immunoglobulins. Once CD40 and CD40L engage, CD40L is rapidly internalized⁶⁰, ensuring that this singular and potent interaction is short-lived.

Many questions about B-cell maturation remain. First, what signals other than CD40 are required for germinal-centre forma-

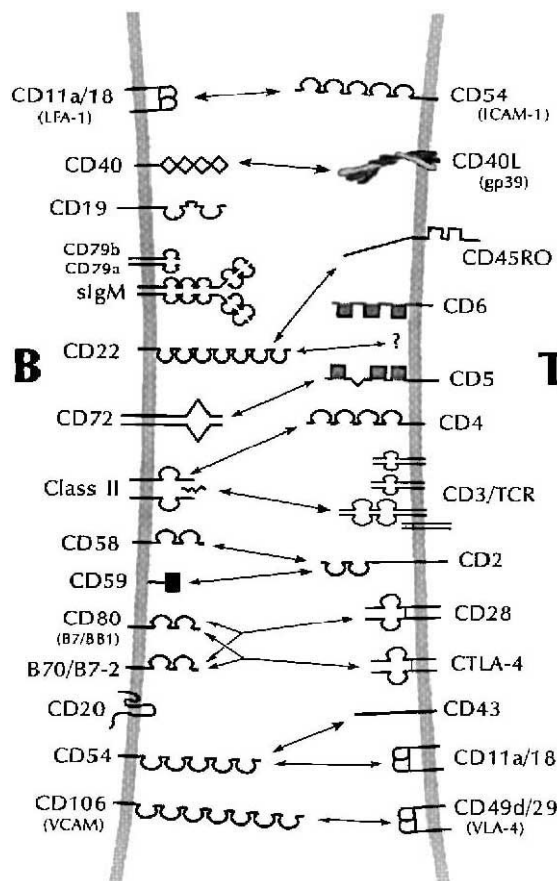


FIG. 2 Adhesion molecules that may mediate interactions between activated CD4⁺ T cells and activated B cells. Domain structures are indicated as follows: immunoglobulin superfamily domains (U), nerve growth factor receptor superfamily cysteine-rich domains in CD40 (X), C-type lectin domains in CD72 (A), scavenger receptor domains in CD5 and CD6 (■), and tyrosine phosphatase domains of CD45 (—).

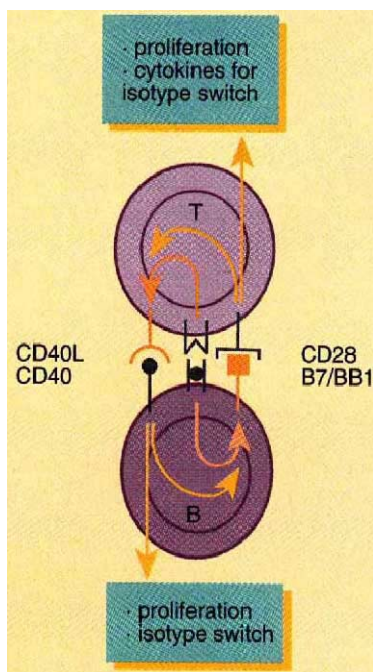


FIG. 3 A reciprocal dialogue between T cells and B cells during T-cell-dependent B-cell maturation. Recognition of class II + peptide by T cells leads to activation of the T cell and new expression of CD40L (Y) and also, via crosslinking of class II, B cells are activated leading to new expression of CD80(B7/BB1) (■). Engagement by CD40L of its receptor CD40 (●) on B cells and by CD80 of its receptor CD28 (L) on T cells amplifies the initial cell activation and leads to B-cell or T-cell proliferation. The activated T cell is induced by CD28 crosslinking to make cytokines needed for immunoglobulin class switching and to express CD40L. The activated B cell is induced by CD40 crosslinking to prepare for cytokine-dependent immunoglobulin class switching and to express CD80 or related molecule B70/B7 (ref. 28). This reciprocal activation and maturation only occurs if either the T cell or the B cell have been previously stimulated to express CD40L or CD80, for example, via an encounter with antigen. Not shown are other receptors, particularly the CD11a/18 (LFA-1) integrin and its ligands such as CD54, which may function to stabilize and modulate the lineage-specific signalling.

tion? Second, what signals induce the somatic mutation of immunoglobulin variable-region genes in B cells, and the subsequent selection of high-affinity antibodies? In addition to their role as antigen depots, follicular dendritic cells also induce this process, while the germinal-centre signals preventing B-cell apoptosis may allow time for it to occur. It remains to be seen, however, what signals direct immunoglobulin class switching, or induce the switched B cells to become plasma cells or memory cells.

Despite this, several basic features of the T-B-cell dialogue have already emerged. First, it is likely that each receptor transmits a message to the cell. Second, other components of the intercellular signalling apparatus, such as cytokines, can affect the expression and activity of cell-surface receptors and, con-

versely, signalling by the receptors can alter cytokine expression. Finally, just as cytokines may provide different signals depending on their context, so cell-surface receptors may transmit different signals depending on the state of B-cell maturation. A complete understanding of the dialogue will thus require an analysis of the way in which the signals delivered by these components interact. □

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Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles

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Now that the genes controlling embryonic patterning have been identified in several model organisms, long-standing questions concerning the evolution of developmental systems are open to investigation. Examination of the expression of *even-skipped* in a variety of insects reveals that insect germ-type designations apparently do not reflect the variations in the mechanisms of segmentation evident throughout insect phylogeny.

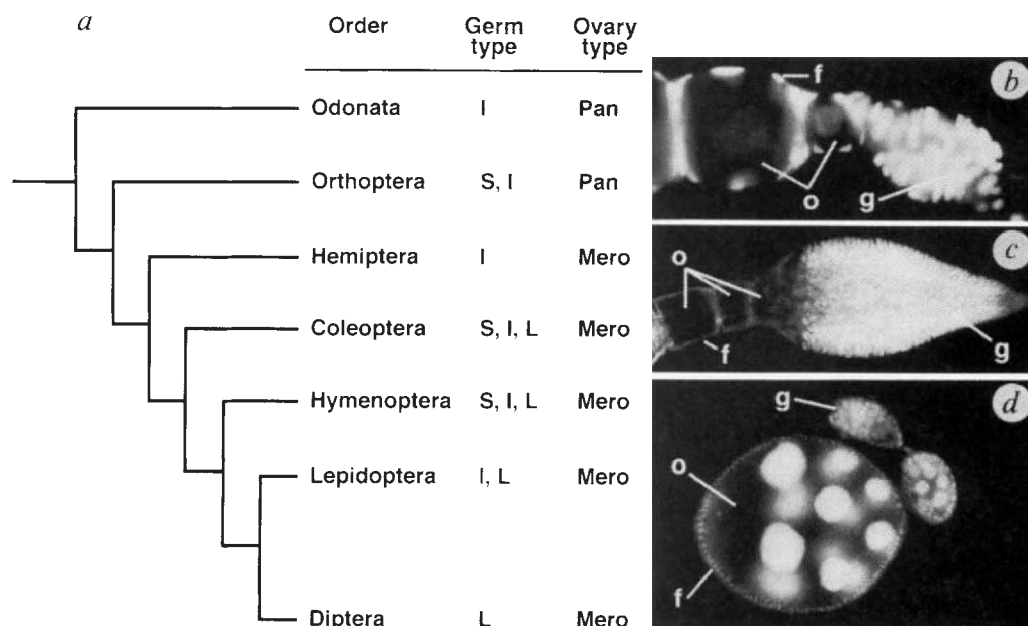
GENETIC and molecular approaches have yielded a wealth of information about the mechanisms of pattern formation in the Dipteran *Drosophila melanogaster*. The anterior–posterior axis of the embryo is first established by gradients of maternal information derived from products transported into the oocyte from nurse cells during oogenesis¹. These gradients initiate the successive expression of zygotic gap, pair-rule, and segment polarity genes, which rapidly subdivide the embryo into progressively smaller units, leading to the nearly simultaneous establishment of segmental pattern throughout the embryo^{2,3}.

There are reasons to believe, however, that not all aspects of the *Drosophila* patterning system are shared by all insects. Even though all insect embryos ultimately establish similar body plans, various experimental perturbations suggest that the initial mechanisms of pattern formation vary considerably among

different insects⁴. A general classification scheme derived from some of these manipulations places insects into three broad categories: long, intermediate and short germ⁴. Extreme long-germ embryos, such as those of *Drosophila*, contain a complete representation of the body plan by the end of the blastoderm state. By contrast, extreme short-germ embryos, such as those of the Orthopteran *Schistocerca americana* (grasshopper), generate all body segments during a post-blastoderm growth phase. Intermediate-germ insects occupy a middle range; segments are established as far posterior as the thorax or anterior abdomen at the blastoderm stage, and the remaining, more posterior segments are formed after gastrulation.

A comparison of a phylogenetic tree of insect evolution with the distribution of germ types (Fig. 1) indicates that the different germ types are distributed throughout the insect orders and that

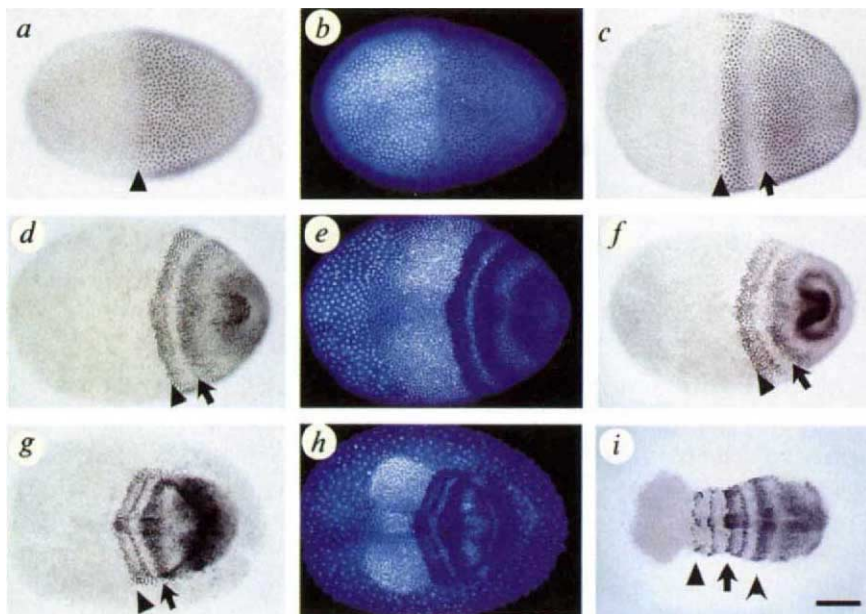
FIG. 1 Relationships of insect orders, germ types, and ovary types. *a*, Phylogenetic tree of a subset of insect orders. The tree is adapted from refs 24 and 25. The germ-type distribution is derived from ref. 4 (S, short; I, intermediate; L long). The ovary type distribution is taken from ref. 26 (Pan, panoistic, no nurse cells; Mer, meroistic, with nurse cells). Germ types do not form separate monophyletic groups, and certain orders include representatives of all three germ types. One general trend is visible: long-germ development appears to be restricted to the more phylogenetically 'advanced' orders, which also all contain meroistic ovaries. *b–d*, Diaminophenylindole (DAPI)-stained ovaries. *b*, The panoistic ovary of the Orthopteran *Schistocerca americana*. Stem cells in the germarium produce oocytes that are not attached to nurse cells. The germinal vesicle of the oocyte transcribes all the maternal mRNA of the egg²⁶. The small, flat cells surrounding the oocyte are the follicle cells, which secrete the chorion of the egg. *c*, The meroistic ovary of the Hemipteran *Oncopeltus fasciatus*. This is referred to as a telotrophic meroistic ovary because the nurse cells, which supply various maternal mRNAs, remain in the germarium and are attached to the anterior end of each developing oocyte



by nutritive cords²⁶. *Tribolium*, *Dermestes* and *Callosobruchus* all possess this type of ovary. *d*, The meroistic ovary of the Dipteran *Lucilia cuprina*. This is an example of a polytrophic meroistic ovary, in which the nurse cells are attached to the anterior end of the oocyte and supply various maternal mRNAs¹, but, unlike in telotrophic meroistic ovaries, the nurse cells move along with the oocyte down the ovariole.

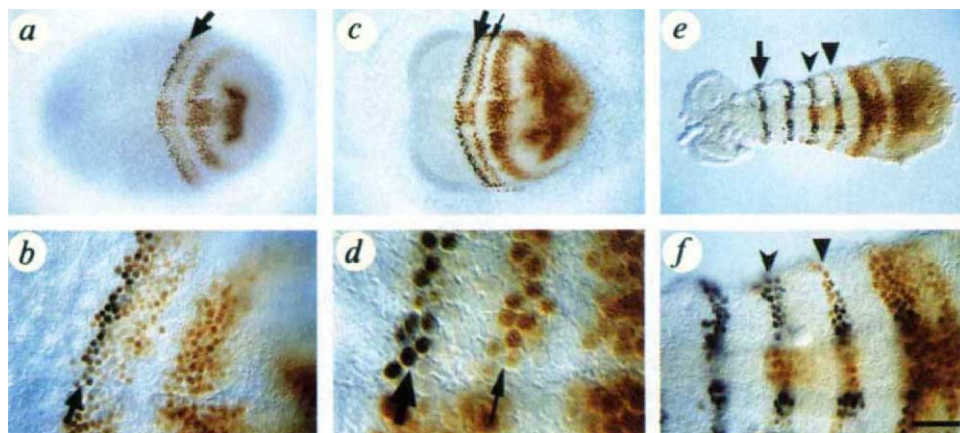
FIG. 2 *Tribolium* *even-skipped* protein patterns. Nomarski (a, c, d, f, g, and i) and fluorescence (b, e and h) images of embryos stained with mAb 2B8 and counterstained with DAPI (DNA stain). Triangles and arrows mark the anterior boundaries of *even-skipped* primary stripes 1 and 2, respectively, from the time that the boundary is first visible to the time its associated primary stripe resolves into secondary stripes. Arrowhead indicates the position of *even-skipped* primary stripe 3. a and b, Initial *even-skipped* expression in the posterior half of the embryo. c, The first *even-skipped* primary stripe forms as *even-skipped* protein disappears from a circumferential interstripe zone. An irregular 'wave' of mitosis then begins and *even-skipped* protein temporarily diffuses from the nucleus to the cytoplasm of mitotic cells (not shown). d and e, The primitive pit begins to form and cells continue to aggregate and divide to form the embryonic germ anlage. Gastrulation now begins at the ventral midline. The second *even-skipped* primary stripe forms as an interstripe appears anterior to the primitive pit. f, *even-skipped* primary stripe 1 begins to resolve into two secondary stripes. g and h, The posterior region of the embryo continues to dive into yolk. At this time, *even-skipped* staining disappears from the extraembryonic serosa, and *even-skipped* primary stripe 1 has resolved into two secondary stripes (1a and 1b). As caudal extension continues, *even-skipped* stripes form in the amnion that are somewhat irregular but are aligned to the stripes in the embryo. i, Embryo dissected free from the egg to expose the more posterior regions. *even-skipped* primary stripes 1 and 2 are both split into secondary stripes (1a, 1b and 2a, 2b) and the interstripe region between *even-skipped* primary stripes 3 and 4 is forming. Anterior is to the left and ventral side is up in all panels. Scale bar, 100 μ m for all panels.

METHODS. Mice were immunized with the previously described *Schistocerca eve/trpE* fusion protein⁸. Of the several hundred hybridoma lines that recognized *Schistocerca even-skipped*, three lines, 3B9, 7H5 and 2B8, were expanded for further study. The staining patterns of mAb 2B8 in *Drosophila* and *Schistocerca* are identical at all stages to published descriptions of *even-skipped* protein expression in these two



insects^{8,12}. Based on the mid-embryonic staining of the characteristic pattern of cells in the dorsal mesoderm, anal pad and nervous system (RP2, aCC, pCC, U, CQ and EL neurons, a medial neuron in the first subesophageal ganglia, and a pair of bilaterally symmetric neurons in the brain)^{27,28}, mAb 2B8 seems to recognize *even-skipped* in the following insects: Diptera, *Drosophila melanogaster*, *Anopheles gambiae*, *Lucilia cuprina*; Hymenoptera, *Camponotus laevigatus*; Coleoptera, *Tribolium castaneum*, *Dermestes frischii*, *Callosobruchus maculatus*; Dermaptera, *Anisotabis annulipes*; Orthoptera, *Schistocerca americana*, *Locusta migratoria* and *Acheta domesticus*. Furthermore, mAb 2B8 staining patterns in *Tribolium* match the distribution of *Tribolium even-skipped* mRNA (J. Parrish, S. Brown and R. Denell, personal communication). Immunohistochemistry has been described¹⁸. Detailed protocols for optimizing egg collection, fixation, and devitellinization for the various insect species are available on request.

FIG. 3 Relationship of *even-skipped* and *engrailed* expression in *Tribolium*. Embryos are stained for *engrailed* in black and *even-skipped* in brown. b, d and f, High-magnification views of the embryos shown in a, c and e, respectively. a, b, At the onset of gastrulation, *engrailed* stripe 1 (wide arrow) appears at the anterior edge of *even-skipped* primary stripe 1. Note that *even-skipped* primary stripe 1 is just beginning to resolve into secondary stripes. c, d, As caudal elongation begins, *engrailed* stripe 1 (wide arrow) and *even-skipped* stripe 1a are coincident. *engrailed* expression has not yet started in *even-skipped* stripe 1b (thin arrow). e, f, In this embryo, *engrailed* stripe 1 is indicated with a wide arrow; *even-skipped* stripes 1a and 1b have faded away; *even-skipped* stripe 2a coincides with *engrailed* stripe 3 (arrowhead) and *even-skipped* stripe 2b coincides with *engrailed* stripe 4 (triangle; *even-skipped* stripes 2a and 2b are most obvious at the midline because *even-skipped* stripes fade from the ectoderm before the mesoderm). *engrailed* stripe 5 is forming at the anterior margin of *even-skipped* primary stripe 3. Anterior is to the left and ventral side is up in all panels. Scale bar: a, 100 μ m; b, 25 μ m; c and e, 75 μ m; d, 10 μ m; f, 30 μ m.



METHODS. *engrailed* expression was detected using mAb 4D9, which recognizes *engrailed* in a wide variety of organisms¹⁸. The inclusion of nickel chloride in the staining reaction was used to generate the black reaction product. Embryos were then washed, stained with mAb 2B8, and reacted without metal ions to reveal the expression of *even-skipped* in brown.

insects with shared germ types do not sort into individual monophyletic groups. Thus although the germ-type classification scheme is an important reminder of the diversity of embryonic development, the simple categorization of long, short and intermediate germ types is insufficient to describe the evolutionary origins of insect pattern formation⁵. Examination of homologues of *Drosophila* segmentation genes in other insects may help establish a more comprehensive framework for describing the evolution of insect pattern formation.

A number of molecular studies have compared *Drosophila* and *Schistocerca* development, in part because *Schistocerca* represents the opposite extreme of several characteristics found in *Drosophila*⁶⁻⁹. Not only is *Schistocerca* a short-germ insect but, in contrast to *Drosophila*, *Schistocerca* is considered to be relatively phylogenetically primitive, it is hemimetabolous, and its eggs are formed without the benefit of maternal input from nurse cells (Fig. 1b-d). Previous experiments compared the *Drosophila* and *Schistocerca* expression patterns of *even-skipped*⁸, a homeobox-containing gene required for proper segmentation and neurogenesis in *Drosophila*^{10,11}. In *Drosophila*, *even-skipped* is expressed during segmentation in a pair-rule pattern and later is a segmentally reiterated subset of neurons^{12,13}. *Schistocerca* shows a similar pattern of *even-skipped* expression during neurogenesis, but no pair-rule pattern was observed during segmentation⁸. This result suggested that short-germ *Schistocerca* embryos, rather than using a pair-rule prepattern, might generate segmental patterns by a different mechanism, such as a system of cell-cell interactions during the growth of the embryo.

The conclusions from comparisons between *Schistocerca* and *Drosophila* may not, however, explain why germ-type differences are also observed within individual insect orders. For example, within the order Coleoptera (beetles), which is considered to be phylogenetically intermediate between Orthoptera and Diptera, species are found that span the entire range of germ types⁴. To study the events that underlie germ-type transitions, we examined *even-skipped* expression in the short-germ beetle *Tribolium*¹⁴, the intermediate-germ beetle *Dermestes*⁴, and the long-germ beetle *Callosobruchus*⁴. In all three beetles, *even-skipped* is expressed in a pair-rule manner, with the only difference being the relative number of stripes formed before, versus after, the onset of gastrulation. Thus, germ-type designations do not necessarily predict the mechanistic details of development: short-germ *Tribolium* is probably more closely related to long-germ *Drosophila* than to short-germ *Schistocerca* with regard to the mechanisms that generate the segmental pattern.

***Tribolium even-skipped* stripe formation**

To examine *even-skipped* expression in the Coleoptera, we used a monoclonal antibody (mAb 2B8) that was generated against the *Schistocerca even-skipped* protein but that also recognizes *even-skipped* in a variety of other insects (see Fig. 2 legend). Before cellularization in short-germ *Tribolium* embryos, *even-skipped* is clearly detectable posterior to 45–50% egg length (100% egg length is the posterior pole of the embryo), and only very low levels are seen in the remaining anterior portion of the embryo (Fig. 2a, b). As development proceeds, *even-skipped* protein is no longer detectable in the anterior half of the blastoderm. During the subsequent course of embryogenesis, eight primary *even-skipped* stripes form from the posterior domain of *even-skipped* expression. The first primary stripe forms as embryonic cells begin to condense towards the ventral surface of the egg (Fig. 2c), and a second primary stripe forms during the onset of gastrulation (Fig. 2e, f). The remaining six primary stripes form sequentially as the embryo undergoes caudal elongation (Fig. 2g, h, i; see also Figs 3 and 6a, c). All the primary stripes arise from a combination of the elimination of *even-skipped* protein from interstripe regions and an increase in *even-skipped* protein levels within each stripe. The interstripe region between two primary stripes is roughly two-thirds the width of a primary stripe. After each primary stripe forms, it resolves

into two thin secondary stripes (a and b) as *even-skipped* protein disappears from a region within the original primary stripe (Fig. 2f-i; see also Fig. 5). These secondary stripes narrow and then fade completely before the morphological appearance of nearby segments. The formation of *even-skipped* primary stripes, the resolution of each primary stripe into secondary stripes, and the eventual disappearance of the secondary stripes, occur sequentially in an anterior-posterior progression along the length of the embryo.

To determine the precise relationship of *even-skipped* stripes with the formation of segmental and parasegmental boundaries, we compared the *even-skipped* and *engrailed* expression patterns. In *Drosophila*, *engrailed* is a member of the segment polarity class of genes and is expressed in the posterior portion of every segment¹⁵⁻¹⁷. Using a monoclonal antibody (mAb 4D9) that recognizes *engrailed* homologues in a wide variety of organisms, previous studies show that this segmental expression of *engrailed* is highly conserved in insects and crustaceans¹⁸, and we used mAb 4D9 to detect beetle *engrailed*.

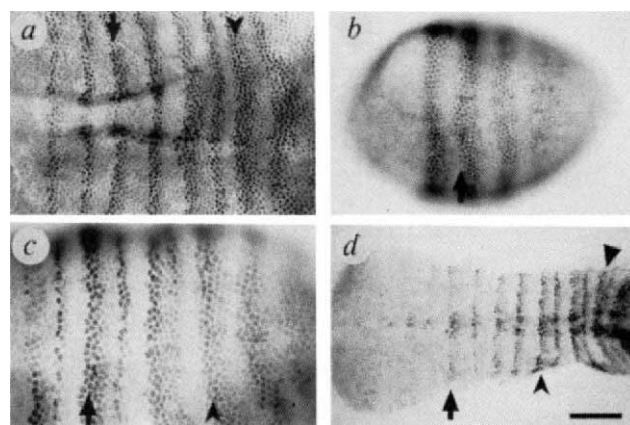
In *Tribolium*, *engrailed* protein stripe 1 (anterior portion of parasegment 1) appears just at the onset of gastrulation; with the exception of the cephalic stripes, the remaining *engrailed* stripes appear sequentially along the embryo as development proceeds. Double labelling for *even-skipped* and *engrailed* proteins reveals that ectodermal cells at the anterior margin of *even-skipped* primary stripe 1 begin to express *engrailed* just as this primary *even-skipped* stripe begins to resolve into its two secondary stripes (Fig. 3a, b). After the first *even-skipped* primary stripe resolves completely into secondary stripes 1a and 1b, *engrailed* stripe 1 and *even-skipped* stripe 1a are coincident (Fig. 3c, d). *engrailed* stripe 2 then appears when cells of *even-skipped* stripe 1b begin to express *engrailed*. As embryogenesis continues, these overlapping patterns of *even-skipped* and *engrailed* are repeated, so that the anterior margin of each *even-skipped* primary stripe prefigures the anterior boundary of each odd-numbered *engrailed* stripe or, in other terms, the parasegmental boundary of each odd-numbered parasegment. The odd-numbered *engrailed* stripes thus correspond to the *even-skipped* 'a' secondary stripes, and the even-numbered *engrailed* stripes correspond to the *even-skipped* 'b' secondary stripes (Fig. 3e, f; Fig. 5). The comparison of *even-skipped* and *engrailed* patterns thus shows that the primary *even-skipped* stripes are pair-rule (because the pattern has a two-segment periodicity) and the secondary *even-skipped* stripes are segmental.

Expression in *Dermestes* and *Callosobruchus*

At the cellular blastoderm stage of intermediate germ *Dermestes*, *even-skipped* protein is detected in all cells posterior to 35–40% egg length. In long-germ *Callosobruchus*, this expression boundary is located at 25–30% egg length. In both of these beetles, as in *Tribolium*, eight primary *even-skipped* stripes form sequentially in an anterior-posterior progression from this posterior domain of *even-skipped* expression, and each of these primary stripes subsequently resolves into two secondary stripes that then disappear (Fig. 4). As in *Tribolium*, the formation of primary stripes involves the elimination of *even-skipped* protein from interstripe regions, and the secondary stripes resolve as *even-skipped* protein disappears from a zone within each primary stripe. Double labelling for *even-skipped* and *engrailed* reveals that the relationship between *engrailed* and *even-skipped* expression in *Dermestes* and *Callosobruchus* is identical to that already described for *Tribolium*: the eight primary *even-skipped* stripes have a pair-rule periodicity and demarcate the anterior margins of odd-numbered *engrailed* stripes, and the secondary stripes are coincident with each *engrailed* stripe (Fig. 5).

A conspicuous difference between these three beetles is, however, seen in the temporal relationship between morphological development and the molecular progression of segmentation. In *Tribolium*, the first primary *even-skipped* stripe appears as cell condensation begins, and a second primary stripe resolves at the

FIG. 4 Expression of *even-skipped* in *Dermestes* (a) and *Callosobruchus* (b, c, d). a, At the onset of gastrulation in *Dermestes*, primary stripes 1, 2 and 3 are in various phases of resolving into secondary stripes (arrow marks stripe 2a). Primary stripe 4 (arrowhead) has just separated from the posterior domain of expression. The folds in the middle of the embryo are the edges of the forming gastral furrow. b, Early in the cellular blastoderm stage, three *even-skipped* primary stripes have already formed in *Callosobruchus* embryos. Low levels of *even-skipped* expression are found in all cells posterior to the third *even-skipped* stripe (the entire posterior region is not visible in this focal plane). The anterior margin of primary stripe 2 is indicated with an arrow. c, Shortly before the start of gastrulation in *Callosobruchus*, *even-skipped* primary stripes 1, 2 and 3 are in various phases of resolving into secondary stripes, whereas primary stripes 4 and 5 are still in their wide, pair-rule pattern. Stripe 2a is indicated with an arrow, primary stripe 4 is indicated with an arrowhead. As primary stripe 2 resolves into secondary stripes, it does so asymmetrically: the 'a' secondary stripe is initially wider than the 'b' secondary stripe. This is the case for the initial formation of all *even-skipped* secondary stripes in all three beetles (see primary stripe 3 in Fig. 3e and f for example). d, At the onset of gastrulation and caudal extension in *Callosobruchus*, *even-skipped* stripes 1a and 1b have faded entirely; primary stripes 2, 3, 4, 5 and 6 are at various points in the process of resolving into secondary stripes; and stripes 7 and 8 have not yet separated from each other. Stripe 2a is indicated by an arrow, stripe 4a by an arrowhead, and stripe 6a by a triangle. Anterior is to the left and ventral side is up all panels. Scale bar: a, 130 μ m; b, d, 100 μ m; c, 50 μ m.



onset of gastrulation. In *Dermestes*, two primary *even-skipped* stripes have formed by the time cells condense toward the ventral side, two more primary stripes have resolved by the onset of gastrulation (Fig. 4a), and the remaining four primary stripes appear during caudal extension. In *Callosobruchus*, the first three primary *even-skipped* stripes are formed by the time cells begin to condense on the ventral side (Fig. 4b), and three additional primary stripes form by the time gastrulation begins (Fig. 4c, d). The seventh and eighth stripes finally separate from one another during caudal elongation.

Comparisons with *Drosophila*

The expression of *even-skipped* and *engrailed*, and the relationship between the two patterns, have been extensively investigated in *Drosophila*^{13,19}, and many aspects of *even-skipped* and

engrailed expression are strikingly similar in *Drosophila* and beetles. In both groups of insects, *even-skipped* is first expressed in a broad domain, pair-rule stripes form by a combination of interstripe repression and stripe activation, and the anterior margin of each *even-skipped* pair-rule stripe prefigures the anterior margin of each odd-numbered *engrailed* stripe¹⁹. The *Drosophila even-skipped* stripes start out approximately six cells wide, with the highest expression within a region of four cells (about one segment width)¹³. Instead of resolving into two secondary stripes as in the beetles, however, the *Drosophila even-skipped* pair-rule stripes narrow to coincide ultimately with the odd-numbered *engrailed* stripes, and weak stripes appear *de novo* that are coincident with even-numbered *engrailed* stripes^{12,13}. The segmental stripes of *even-skipped* alternate in intensity in *Drosophila* but are of equal intensity in the beetles. The resolution of *even-*

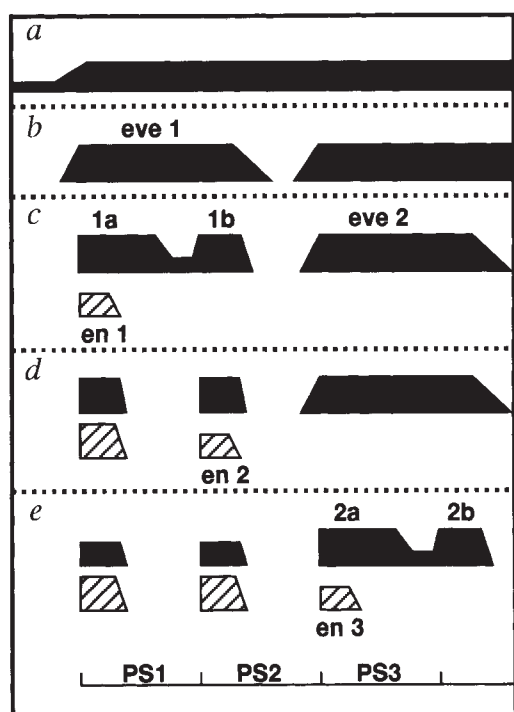
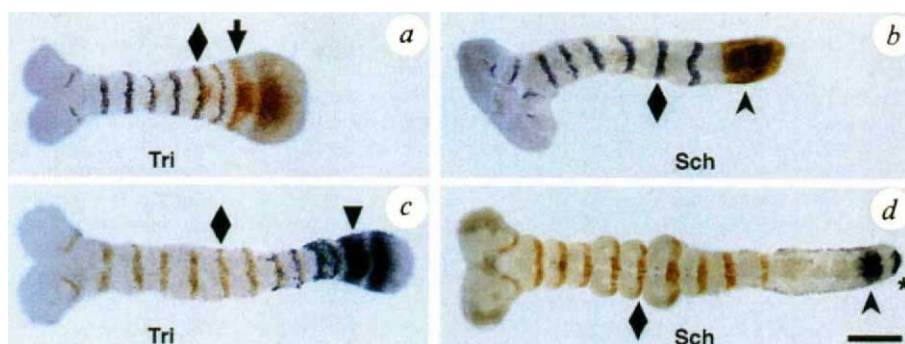


FIG. 5 Diagram of the relationship between *even-skipped* and *engrailed* expression in all three Coleoptera. Solid blocks represent *even-skipped* expression, and hatched boxes represent *engrailed* expression. a–e, Successively later stages during the establishment of *even-skipped* and *engrailed* patterns in the anterior regions of the embryo. Parasegment boundaries (PS) are indicated at the bottom of the diagram. The heights of the boxes reflect the approximate levels of protein expression. The pattern shown here continues down the length of all three beetle embryos as development proceeds.

FIG. 6 Comparison of *even-skipped* and *engrailed* patterns in *Schistocerca* and *Tribolium* during abdominal segmentation. *Tribolium* (Tri; a and c) and *Schistocerca* (Sch; b and d) embryos stained for *engrailed* in black and *even-skipped* in brown (a and b) or *even-skipped* in black and *engrailed* in brown (c and d). In all four panels, a diamond marks *engrailed* stripe 5 (second thoracic segment). The *Tribolium* and *Schistocerca* embryos in a and b are at similar stages of generating segments because both have six *engrailed* stripes. Likewise, those in c and d are at a similar stage because both have just started to form the ninth *engrailed* stripe (observed by *even-skipped* stripe 5a in the *Tribolium* embryo). In the *Tribolium* embryos, pair-rule (primary) stripes of *even-skipped* are clearly visible. The arrow in a indicates the position of primary *even-skipped* stripe 4. In c, a triangle marks primary *even-skipped* stripe 6. Primary stripes 7 and 8 are still fused, and stripes 4a and 4b have almost completely faded away (the irregularly positioned black cells in 4a and 4b are in the amnion overlying the embryo). In the *Schistocerca* embryos, *even-skipped* is expressed in a posterior domain (arrowhead in b and d), but no pair rule pattern of stripes is seen. The staining at the posterior tip in d is the start of expression in the anal pad (asterisk), a structure that expresses *even-skipped* in all insects examined, and the isolated *even-skipped* expressing cells in the region of the thorax are the ganglion mother cells that produce RP2 neurons. These photographs show that both *Schistocerca* and *Tribolium* embryos increase in length as they develop. As the *Schistocerca* abdo-



men changes in length, it maintains a constant width, but as the *Tribolium* abdomen increases in length, it decreases in width. *Tribolium* embryos appear to 'pinch down' as sequential *engrailed* stripes form. Preliminary studies suggest that *Tribolium* embryos undergo phases of regional proliferation followed by convergent extension and that *Schistocerca* embryos also exhibit phases of regional proliferation, but without convergent extension (N.H.P., unpublished observations). It is possible that at least part of the shape changes seen in *Tribolium* are due to the same type of cell intercalations that occur during *Drosophila* germ-band extension. More detailed cell lineage analysis will be required to accurately establish the relationship between 'growth' and patterning in these insects. Anterior is to the left and ventral side is up in all panels. Scale bars: a, c, 100 μ m; b, d, 225 μ m.

skipped primary pair-rule stripes into secondary segmental stripes in the beetles is reminiscent of a similar transformation described for the stripes of the pair-rule gene *paired* in *Drosophila*²⁰.

All the beetles generate eight pair-rule stripes of *even-skipped*, but only seven are seen in *Drosophila*. The seventh *Drosophila* *even-skipped* stripe is wider than the rest^{12,13}, however, which is consistent with the idea that the more posterior segments of *Drosophila* have become fused together. Interestingly, *even-skipped* disappears from the posterior end of all three beetle embryos at the same time that the anterior margin of *even-skipped* primary stripe seven becomes visible (Fig. 6c). Thus, beetle *even-skipped* primary stripes 7 and 8 start out fused and separate only later in development.

Short-germ *Tribolium* and *Schistocerca* compared

We have examined younger (8–10% of development) *Schistocerca* embryos than previously⁸ and find that *even-skipped* is expressed by all cells in the gastral groove and all cells in the posterior two-thirds of the embryo at this stage. As development proceeds, expression rapidly disappears from the proliferating thoracic region and then from the gnathal region, leaving expression in a more posterior domain, as described previously⁸. This posterior expression is highest in the mesoderm but the monoclonal antibodies reveal that *even-skipped* protein is also present in the overlying ectoderm (Fig. 6b, d). No pattern of pair-rule stripes is seen and no overlap is detected with the developing *engrailed* stripes.

Figure 6 shows the striking differences in the relationship of *even-skipped* and *engrailed* expression in *Schistocerca* and *Tribolium* embryos. As already described, *even-skipped* is expressed in pair-rule and segmental patterns in *Tribolium* before the establishment of *engrailed* stripes (Fig. 6a, c). In *Schistocerca*, *even-skipped* is expressed in a posterior domain, but no pattern of pair-rule stripes resolves from this domain (Fig. 6b, d). In addition, both *engrailed* expression and the morphological formation of segments begin in the thorax (parasegments 4 and 5) and spread both anteriorly and posteriorly⁶. By contrast, in *Tribo-*

lium, *engrailed* stripes and the morphological development of segments begin in parasegment 1 and spread posteriorly.

Discussion

To analyse the evolution of insect pattern formation, we examined *even-skipped* expression in three beetle species that span the range of germ types, from short-germ *Tribolium* to intermediate-germ *Dermestes* to long-germ *Callosobruchus*, and compared these patterns with *even-skipped* expression in long-germ *Drosophila* and short-germ *Schistocerca*. We infer two major conclusions from our results: (1) germ-type designations do not necessarily correlate with a particular mechanism of pattern formation, but (2) germ-type designations, at least within the Coleoptera, do accurately predict the temporal aspects of segmentation.

In all three beetles, *even-skipped* is expressed in identical spatial patterns. Eight *even-skipped* pair-rule stripes are established from a posterior domain of *even-skipped* expression by the elimination of *even-skipped* in interstripe regions; these pair-rule stripes then resolve into segmental stripes. In addition, the relationship between *even-skipped* and *engrailed* patterns is identical in all three beetles. This suggests that although each beetle belongs to a different germ type, *Tribolium*, *Dermestes* and *Callosobruchus* seem to use identical mechanisms of pair-rule patterning, at least as determined by the analysis of *even-skipped* and *engrailed* expression.

The expression of *even-skipped* and its relationship to *engrailed* expression are similar in *Drosophila* and beetles: in both insects, the anterior margins of *even-skipped* pair-rule stripes demarcate the anterior boundary of odd-numbered parasegments, and the segmental secondary stripes align with each *engrailed* stripe. Because the function of beetle *even-skipped* has not been examined genetically, however, it is not yet known whether *even-skipped* is required for the expression of all *engrailed* stripes in beetles as it is in *Drosophila*¹³. Despite the striking similarities in *even-skipped* expression patterns, some aspects of pair-rule patterning are likely to be different between *Drosophila* and Coleoptera. For example, the differences in establishment of

secondary (segmental) *even-skipped* stripes in *Drosophila* and Coleoptera may indicate there are slight differences in the pair-rule gene interactions in the two groups of insects. Thus, our analysis of *even-skipped* expression suggests that long-germ *Callosobruchus* is more closely related to short-germ *Tribolium* than to long-germ *Drosophila* in regard to the mechanisms of pair-rule pattern formation, consistent with the phylogenetic relationships of these three insects.

In short-germ *Schistocerca*, *even-skipped* is expressed in a posterior domain, but no pattern of pair-rule stripes resolves from this domain and no overlap is detected between *even-skipped* and *engrailed* expression⁸. In addition, pair-rule patterns for *fushi tarazu* are seen in *Drosophila* and *Tribolium* (S. Brown and R. Denell, personal communication) but not in *Schistocerca* (R. Dawes and M. Akam, personal communication). Thus, the establishment of segments in *Schistocerca* may not involve pair-rule pre patterning. The available data indicate that short-germ *Tribolium* is more closely related to long-germ *Drosophila* than to short-germ *Schistocerca* in regard to the use of pair-rule patterning mechanisms. Taken together, our results indicate that germ-type designations do not correspond to specific mechanisms of pattern formation. Closely related short-, intermediate- and long-germ insects (*Tribolium*, *Dermestes* and *Callosobruchus*) can use similar segmentation mechanisms, whereas two distantly related short-germ insects (*Tribolium* and *Schistocerca*) may use different segmentation mechanisms.

Our results do show, however, that germ-type designations accurately predict the overall temporal aspects of beetle segmentation, as assayed with molecular markers. The expression of pair-rule and segment polarity genes in *Drosophila* shows that the entire segmental pattern is established by the onset of gastrulation^{2,3}. Based on *even-skipped* and *engrailed* expression, segmental patterning in *Tribolium* has only proceeded as far as the gnathal segments of the head by the start of gastrulation (Fig. 2d). At a similar stage in *Dermestes* development, segments have formed as far posterior as the anterior abdominal region. In *Callosobruchus*, all but the posterior-most abdominal segments are established before the onset of gastrulation. The analysis of *engrailed* expression in *Schistocerca* indicates that all the segments of the gnathal, thoracic and abdominal regions are established after gastrulation⁶. It should be noted that there has been some controversy on the assignment of *Tribolium* as a short- or intermediate-germ insect¹⁴, but our results on *engrailed* expression clearly support the classification of *Tribolium* as a short-germ beetle. It will be interesting to examine *even-skipped* and *engrailed* expression in certain Lepidoptera, whose morphological development is reminiscent of intermediate germ types but for which the results of experimental perturbations are consistent with a long-germ designation⁴.

We postulate that the transitions between short-, intermediate- and long-germ development within the Coleoptera is simply the result of heterochrony. That is, the same pair-rule patterning

mechanism generates the segmental pattern in all Coleoptera, but changes in the relative timing of morphological development and the molecular process of segmentation have given rise to the continuum of germ types within the Coleoptera. The germ-type differences between *Drosophila* and *Schistocerca*, on the other hand, are more likely to have resulted from changes in the mechanisms that generate anterior-posterior patterns.

Previous results showed that pair-rule patterns of *hairy* expression are seen in *Drosophila* and *Tribolium*¹⁴. From this it was suggested that pair-rule patterning is used in *Tribolium* and that pair-rule patterning is a fundamental component of segmentation in all insects¹⁴. Our results support the first conclusion but not the second. Although we cannot absolutely rule out pair-rule patterning mechanisms in *Schistocerca*, our results argue that comparisons between long-germ *Drosophila* and short-germ *Tribolium* cannot be used to predict properties possessed by all insects. Similarly, caution should be applied in interpreting the results from *Schistocerca*. Although *Schistocerca* development has been put forward as a model for the 'primitive' mode of insect pattern formation^{6,21}, several other species from phylogenetically primitive orders have been described as intermediate germ types, and, more importantly, the results of embryonic perturbations in some of these insects suggest that they develop somewhat differently than *Schistocerca*⁵. We are currently expanding our studies on *even-skipped* to some of these phylogenetically primitive intermediate-germ insects, as well as to crustaceans, to compare their development to *Schistocerca* and to gain a better understanding of the ancestral mode of insect pattern formation. Several unusual species of phylogenetically advanced orders also deserve further investigation, particularly members of the Hymenoptera in which multiple embryos develop from a single egg.

Although germ types do not correlate with potential mechanisms that establish segments, the limited data suggest a correlation between segmentation mechanisms and ovary types. In addition to Diptera and Coleoptera, evidence for pair-rule patterning has been seen in Hymenoptera²² and Lepidoptera²³, all insects with meroistic ovaries (Fig. 1c, d). Pair-rule patterns have not been observed in *Schistocerca*, an insect with panoistic ovaries (Fig. 1b). Further studies will be required to determine whether ovary types truly limit the systems of pattern formation, and an analysis of phylogenetically advanced groups, such as fleas, that have reverted back to panoistic oogenesis would be particularly interesting. It is worth stressing that even though the general classification scheme of germ types may not correlate with segmentation mechanisms, the data collected from embryonic perturbations studies are invaluable, especially in systems where genetic analysis is not yet possible. Ultimately, discerning how insect segmentation has evolved will require genetic, molecular and embryonic perturbation data from species spanning the range of insect phylogeny. □

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Mechanism of a morphology transition in ramified electrochemical growth

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RAMIFIED patterns are common in nature, and have been much studied in the context of non-equilibrium growth and aggregation phenomena¹⁻⁴. Processes that are known to produce ramified growth may lead to a range of morphologies, depending on experimental parameters such as growth speed. The mechanism of the transitions between different morphologies is not fully understood¹⁻⁴. Electrochemical deposition of ramified deposits⁵⁻²⁹ is often regarded as a model system for studying two-dimensional pattern formation. Here we present experimental results which allow us to propose a mechanism for the transition between fractal-like, ramified growth and rectilinear, filamentary growth of electrodeposits. We show that electroconvection of the metal ions in solution induces physical displacements of the growing branches, which results in fanning and splitting of the tips. At sufficiently high growth speeds, breaking of the symmetry with which this fanning occurs can lead to a change in growth morphology. We suggest that mechanical motions and disturbances may play a role in other pattern-forming systems.

In quasi-two-dimensional electrochemical deposition, a cell is formed by sandwiching two electrodes and a solution of a salt (here copper sulphate) between two glass plates. On imposing a current, a tree-like deposit forms by reduction of the metal ions. On increasing the current density (from 5 to ≥ 20 mA cm⁻²), the morphology of the deposit changes from a ramified deposit (Fig. 1a) to a more regular deposit made of rectilinear filaments with small side-branches (Fig. 1b).

It has been shown recently that space charges at the tips of the branches²⁰ trigger a convective motion in the electrolyte^{22,23}, which is given, to a first approximation, by $\mathbf{v} = \nabla \times \Psi$, with $\Psi = (0, 0, \psi)$ and

$$\psi = v_a x + \frac{s}{48\pi\rho\nu} \int_{k=-\infty}^{k=+\infty} \frac{x - kb}{r_k^2} dk \quad (1)$$

Here ρ is the density and ν the viscosity of the liquid, f is the

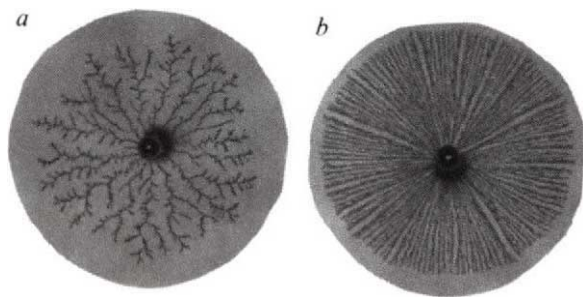


FIG. 1 a, A ramified deposit obtained in circular geometry with a cell thickness of 0.2 mm and 0.01 M copper sulphate solution. The anode is a copper ring of 15 cm diameter and the centrally located cathode is a copper wire. The growth is stopped at a radius of 4 cm. In this experiment the current density (midway) is of the order of 5 mA cm⁻². b, A dense radial pattern obtained with a current density of the order of 30 mA cm⁻². The branches are more rectilinear and they have very few side-branches.

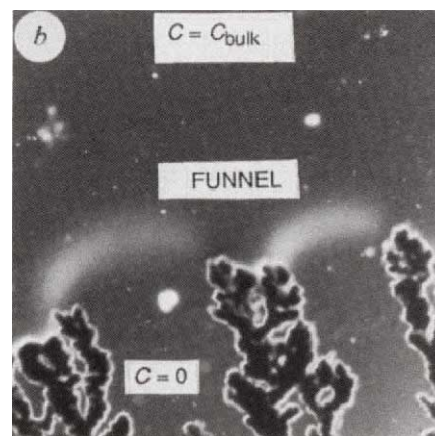
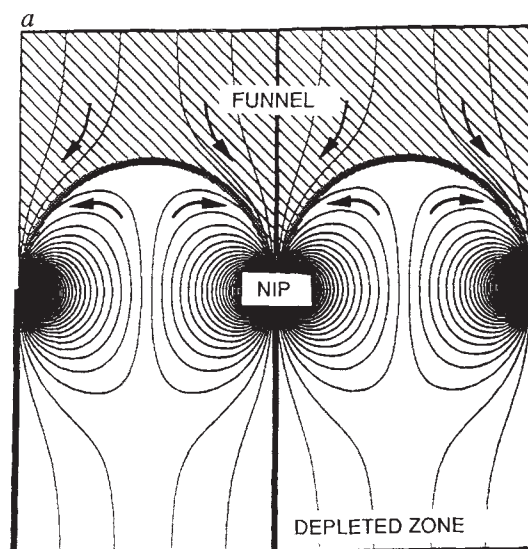


FIG. 2 a, The streamlines in the moving frame of the tips, in the idealized situation of growth of an infinitely thin comb, where a pair of contra-rotating vortices are expected to exist between the tips. The upper part of the vortices defines a line which makes an arch going from one tip to the next. The inner part of the vortices is ion-depleted. The concentrated solution is found only above the arch, where the concentration is almost constant. The copper ions are brought to the tip through some kind of a funnel. In practice, the fluid speed is not infinite in the funnel. Oil droplets were used as tracers in order to see the fluid flow, and to measure the actual speed u in the nip. For a field E of 20 V cm⁻¹ we found $u \sim 1-3$ mm s⁻¹, and for $E \sim 2$ V cm⁻¹, $u \sim 0.1-0.3$ mm s⁻¹. These values can be checked independently by considering the flux of liquid through the nip, $u \sim v_a(b/n)$, where n is the width of the nip, and v_a is the spread of the anions. We have observed $b/n \sim 20$. $E \sim 20$ V cm⁻¹ implies $v_a \sim 2 \times 10^{-1}$ mm s⁻¹, so $u \sim 2.5$ mm s⁻¹, which is consistent with the direct measurement. The Reynolds number is 0.5-5 in the 'straight branches' regime explored here. This is neither large nor small. It should be noted that the fluid is not flowing along a smooth interface: the complicated structure of the deposit breaks the streamlines and makes the flow near the branch all the more irregular as the fluid through the nip (and the branch) is faster. (Garik and coworkers have independently proposed the existence of cellular convection in electrochemical deposition²⁶, on the assumption that Marangoni convection would be important in this kind of experiment. b, Image in interferential contrast (ref. 22) showing the concentration gradient. The zone between the branches is of uniform colour, representing a constant concentration, C , of zero. The transition between this depleted zone and the bulk solution ($C = C_{bulk}$) has the shape of an arch (see text and ref. 22). The thickness of the funnel between these arches is proportional to $1/u$. In the straight-branches regime, the funnel is narrow. If a branch finds itself tilted laterally inside the vortices, it stops growing because it moves into a solution depleted of metal ions.

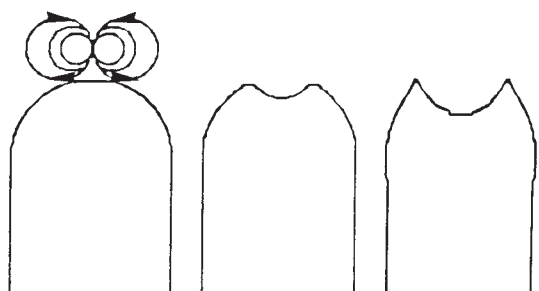


FIG. 3 Schematic diagrams showing why a branch is given a fan-like shape by the incoming fluid: assuming that the branch is simply a collection of small crystallites which float freely in the solution, one can determine the evolution of a tip (here a bar with hemispherical apex) by letting the crystallites drift in the fluid flow given by equation (1). The incoming fluid splits the branch into two cusps. The actual density of the deposit is $\sim 10^{-4}$ times the density of compact metal, which, to some extent, justifies the assumption of free drift of the crystallites. The electric field will be stronger at the cusps, which will tend to generate their own vortices. Branching will persist if a new pair of vortices can be formed between the two nascent cusps. The gap between the cusps increases with increasing fluid velocity.

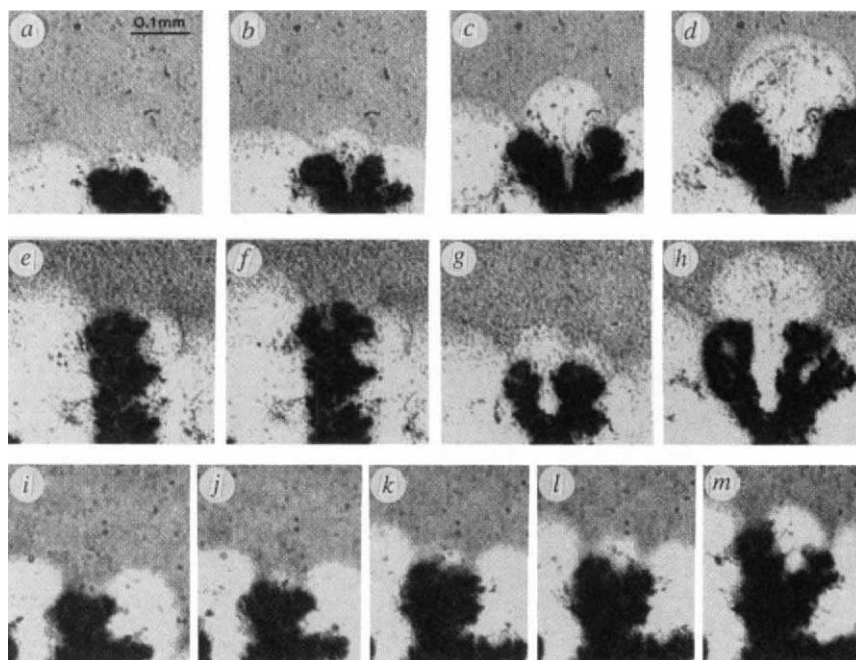
force acting on the liquid, b is the distance between branches, v_a is the speed of the anions, s is the thickness of the cell, r_k is the distance to the tip of a given tip k (equation (1)) assumes that there is an infinite number of parallel branches if there are only N branches, k goes from 1 to N) and x is the direction perpendicular to the tips. This gives the streamlines shown in Fig. 2a. The ions inside the vortices are swept towards the tips so that, in the steady state, the vortices are completely depleted of both cations and anions. Within a funnel-like region between the vortices, however, the concentration of ions remains almost equal to that in the bulk solution (Fig. 2). These features have

been observed experimentally²³. The actual fluid field and the concentration maps are very close to the predicted ones: the approximation given by equation (1) is very good, except at the tips themselves where the fluid flow is perturbed by its journey through the branch. Also, the width of the concentrated zone that encompasses the nip of this funnel must vary $\propto 1/u$, where u is the fluid speed in the nip²³.

We assume that (1) The inner part of the vortices is ion-depleted; (2) the nip is thinner at faster growth speeds; (3) the fluid flow is oriented towards the tip and is given by equation (1); and (4) the deposit is extremely porous at small scales. (It is known that these deposits are roughly twice as dense as the metal in the solution, that is 10^{-4} times as dense as compact metal²¹. (See also Fig. 1e of ref. 3).

As the branch is made of an extremely loose bunch of crystallites, the strong convective flow that is observed can deform the active tip. The streamlines are circles flowing inwards, so they tend to advect the left part of the tip towards the left, and the right part of the tip towards the right ('fanning' of the branch). This generates cusps on both sides of the branch. A simple simulation (Fig. 3) explains schematically how the fanning proceeds in an idealized situation. According to ref. 20, the new cusps generate larger space charges because the field is larger at the cusps; according to refs 22 and 23 this generates new vortices. If the two cusps survive, the zone between the new tips will be emptied of both cations and anions by the new vortices, and there will be a new concentration map with an arch between the two new tips. At low growth speeds the fluid flow is slow, the fanning of the tip is slow and the active zone is quiet. Also, the funnel ahead of the branch is wider. The generation of new vortices between the two cusps is possible because (1) the fluid does not break the symmetry of the tip-splitting process: the cusps move and grow apart symmetrically, the convective forces at their tips are of comparable magnitude and they progressively generate a new pair of vortices; (2) the cusps do grow while they fan because they remain in a concentrated zone. Eventually, the pattern is ramified.

FIG. 4 a-d, e-h and i-m, Three sequences showing tip-splitting. Cell dimensions, $1\text{ cm} \times 1.5\text{ cm} \times 0.1\text{ mm}$; solution, 0.02 M copper sulphate; field, 2 V cm^{-1} . In this instance, ramification occurs. The fluid flow is visualized with oil droplets (ESSO cutwell 42) added to the solution. Because the vortices sweep the droplets towards the tips, there are fewer droplets inside the vortices, which appear lighter. In each strip, the initial tip is slightly opened by a physical displacement. This is especially clear in (a-b and e-f), where a groove appears, separating the two cusps. The two cusps continue to grow into the zone of high ion concentration at the tip. A new arch then forms between the new cusps. As tip-splitting proceeds, the two cusps move apart physically. Eventually the arch becomes well established and the two branches keep on growing. The time lapse between a and b, and between e and f is $\sim 2\text{ s}$. The last two images in the top and middle strips are taken later, when the arch is well established (c and g $\sim 5\text{ s}$ afterwards, and d and h $\sim 10\text{ s}$ afterwards). In the bottom strip the time interval between each image is the sequence (i-l) is $\sim 1\text{ s}$. Image m is taken after $\sim 5\text{ s}$. Notice in the middle strip that the tip splitting e-f-g was followed immediately on the right-hand tip by another tip-splitting attempt which eventually was not successful. In images g and h one clearly observes that the second tip-splitting, which occurred on the right-hand tip, was accompanied also by a physical displacement and the formation of a groove between the cusps.



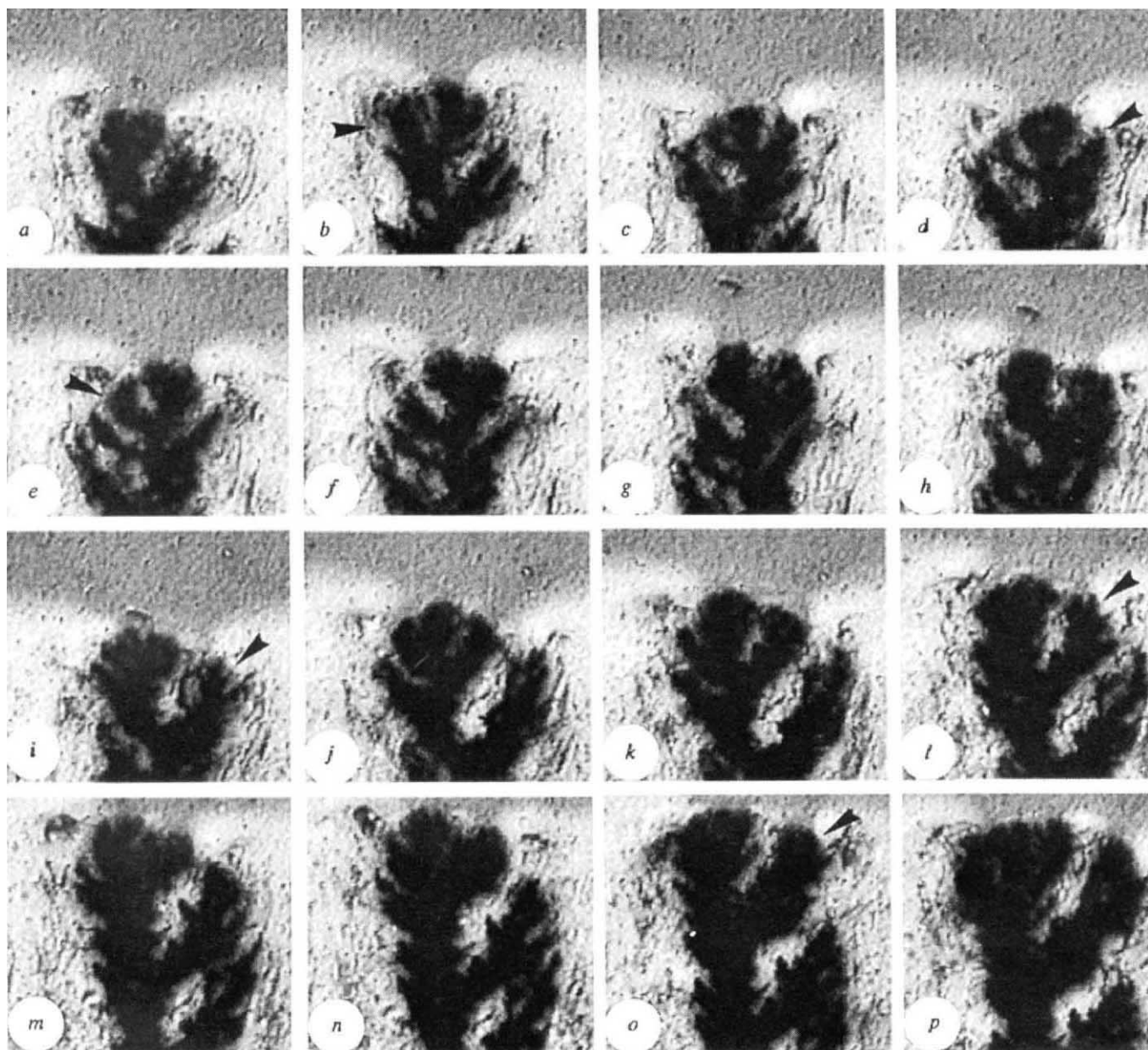


FIG. 5 A growth with an electric field of 20 V cm^{-1} . The sequence of images a–p shows 20 s of growth of a single tip. The time interval between branches is $\sim 1 \text{ s}$. Arrows indicate some of the side 'hairs' which were formed in the active zone, and which have been pushed aside. The convective motion deflects the 'hairs' to either side, where the ion concentration is depleted: they therefore stop growing. The gap between the pair of cusps is larger under these conditions because the

fluid speed is higher and therefore the deforming force is stronger. Also, the growing cusps are tilted laterally, as a consequence of fanning at the tip. In images h–l, one sees that one of the small cusps survives longer, but is screened eventually. In some images (especially a–d) trajectories traced by filaments of flocculated oil droplets can be seen, which show that the fluid flow is not perfectly laminar.

At higher growth speed, the fluid flow is faster, fluctuations in the active zone are larger, and the 'nip' of the funnel is thinner. The generation of new branches is more difficult, for two reasons. First, the random fluctuations of the active zone break the symmetry of the tip-splitting process: of two cusps which the fluid flow tends to form, the one that is furthest forward follows the funnel, goes ahead and remains in the concentrated zone. Second, the cusp which is pushed laterally finds itself in a depleted zone and stops growing. The overall effect is that, at larger growth speeds, there is a change in growth morphology: the branches are straighter and they exhibit small side 'hairs' which are, in our explanation, branches that grew from cusps but failed to survive.

Our experimental apparatus has been described in refs 21 and 22: it enables the convection and concentration of a solution to be visualised by means of interferometric contrast. We have followed the tip-splitting event at slow (Fig. 4) and fast (Fig. 5) growth: the ramified and rectilinear cases respectively. Three typical sequences are represented in Fig. 4 (a–d, e–h and i–m) which show the branch and the fluid flow during fanning. The vortices show up as lighter in colour, and the fluid outside the vortices shows up as grey. In each strip, the formation of new vortices may be seen between the new cusps, creating a new arch joining the small cusps, which both survive. The physical displacement of the nascent tips (especially Fig. 4a–d and Fig. 4e–h) may be clearly seen. In Fig. 5 (which is a sequence of 16

frames, with ~ 1 s between frames) one observes many offshoots of the growing tip which do not survive because the growth is too fast. The cusp which remains in front of the existing funnel rushes ahead, while the cusp which finds itself to one side stops growing; this is because the inside of the vortices is ion-depleted (see also Fig. 2b). (We have also observed that if the funnel is tilted, the symmetry is broken on a well defined side).

It is known in other growth processes that a local anisotropy may induce a macroscopic change to a dendritic structure¹⁻⁴. The transition reported here is an example of another local phenomenon inducing a macroscopic effect from ramified to rectilinear branches. We have also shown that stronger fluctuations can lead, surprisingly, to more regular patterns. Note that the main features of this transition cannot be obtained from a

Mullins-Sekerka⁴ type analysis. This suggests that linear stability analyses may be misleading if they lack a description of the microscopic phenomenon of branching. Finally, as it is a general trend of growth phenomena that the concentration gradients are sharper and the fluctuations are stronger at faster growth speeds, we believe that mechanisms analogous to the one reported here must be considered whenever mechanical motion of the deposit occurs. In other systems, steric hindrance is a possible source of such motion. We have observed it in electrochemical deposition, in instances where vortices are not visible. We have also observed another type of fanning motion probably due to electrostatic repulsion. The fluctuations due to these motions provoke symmetry breaking of the tip-splitting which is qualitatively the same as in the case of fluctuations due to fluid motion. $\square$

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Formation of a mesh-like electrodeposit induced by electroconvection

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ELECTRODEPOSITION of metals such as copper and zinc from solutions of their salts may give rise to ramified metal deposits which show a range of growth morphologies¹⁻⁹. Our understanding of the factors that determine growth morphology is still very limited, in part because different morphologies may be observed even under similar growth conditions^{4,5}. It is thought⁹ that uncontrolled convective processes at the tips of the deposit branches may play a role in these discrepancies. Here we show that convective effects, which we can visualize directly, in the electrodeposition of iron from FeSO₄ solution, can generate a mesh-like pattern, a morphology that has not been reported previously. Convection can be diminished by altering the pH, whereupon we see a transition to a dense branching morphology. Our results show that convective effects do indeed play an important part in determining pattern selection during electrodeposition.

The metal deposit formed in electrodeposition from acidified FeSO₄ aqueous solution film (Fig. 1) and its evolution process (Fig. 2) are studied using an *in situ* observation method. Unlike the fractal-like or dendritic patterns observed previously^{1-6,10}, in

our experiment the neighbouring tips of the deposit branches approach each other and form a mesh-like pattern. In order to find out the possible mechanism behind this phenomenon, interference contrast microscopy, a method capable of mapping gradients in concentration, is employed to investigate the concentration profile near the growing interface (Fig. 3). Bright arches, which signify local concentration gradients, are found connecting the neighbouring branches. Moreover, the tips of the deposit branches approach each other along the arch. The curvature and the contrast of the arch decrease during this process (Fig. 3a-d). We have found that observation of this phenomenon requires sufficient H₃O⁺ concentration in the electrolyte solution: if no sulphuric acid is added to the FeSO₄ solution initially, the mesh-like pattern is not observed, the morphology instead being densely branched.¹¹ Additionally, in electrodeposition of acidified FeSO₄ solution, when the mesh-like pattern grows for some time, a morphology transition from a mesh-like pattern to a dense branching morphology (DBM) (Fig. 4) can be observed. Measurements indicate that the morphology transition corresponds to an increase in pH of the electrolyte solution, owing to generation of hydrogen gas. When the morphology changed to DBM, the arches connecting the neighbouring branches disappear and a more uniform envelope emerges. The tips of the branches do not then approach each other.

To study the stream pattern around the growing tips, we used transmission optical dark-field microscopy and interference contrast microscopy to trace the movement of small particles in the electrolyte solution. We find that during mesh formation, the particles flow towards the tips of the branches and those trapped in between the branches flow in circles. In contrast, during the growth of DBM, the small particles move slowly towards the deposit branches and do not circulate. The observations indicate

that connection of the branches is related to interbranch convection.

Recently, Fleury, Chazalviel and Rosso proposed⁹ an electro-convection model to explain the arch-shaped concentration distribution in front of the deposit branches. In this model, positive charges are assumed to exist in the liquid just in front of the tip of the branches and the growth speed of the deposit is the speed at which the anions are withdrawn from the deposit. A coulombic force is assumed to act on the deposit tips owing to this local accumulation of electric charge. Two vortices between the neighbouring tips of branches are expected, shown schematically

in Fig. 3e. The largest loops of the vortices form an arch (the dashed line in Fig. 3e), which separates two zones: a depleted zone (the area below the arch) and a zone with constant concentration (above the arch). In the area of constant concentration, the stream lines converge to the tip of branches and there is no circulation; in contrast to the upper region, in the depleted area the flow is circulating. So the electrolyte in the region above the arch cannot enter the lower part by flow, that is, the boundary between these two zones is very sharp if diffusion is ignored.

In reality, however, the ion diffusion widens the virtual interface between the two zones. The arches connecting the neigh-

FIG. 1 The morphology of the iron deposit observed during electrochemical deposition from acidified FeSO_4 aqueous solution films. The growth pattern and the evolution of morphology is observed using an optical microscope and recorded by either a video system or a camera. METHODS. The cell for electrochemical deposition consists of two closely spaced microscope glass slides, sandwiching the electrolyte. The thickness of the film is controlled by two mica spacers, varying in thickness from 10 to 120 μm . Two straight, parallel electrodes, (made of iron with purity 99.997% (anode) and a pencil core with rectangular cross-section (cathode)) are fixed on the bottom slide, 22 mm apart. This cell is almost the same as that reported previously¹³, except that the gap between the cathode and the edge of the upper glass plate is slightly larger. In this way the hydrogen generated at the beginning of the experiment can be released easily, otherwise the later growth of the iron deposit will be completely blocked by the hydrogen bubbles. Analytical grade FeSO_4 (99.5% pure) is used to give a 0.5 M solution; sulphuric acid is added in drops to give a pH of ~ 2.0 . The applied voltage across the two electrodes is 4.000 ± 0.001 V, kept constant during the experiment. (The voltage should not be too high during growth in order to prevent the generation of too many hydrogen bubbles.) The electrodeposition is carried out at room temperature ($\sim 20^\circ\text{C}$).

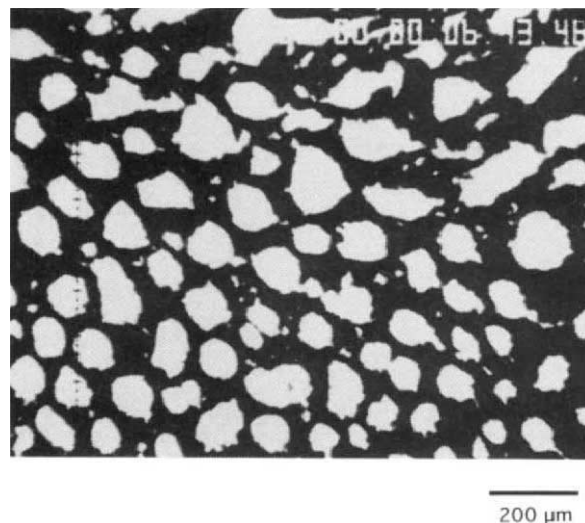
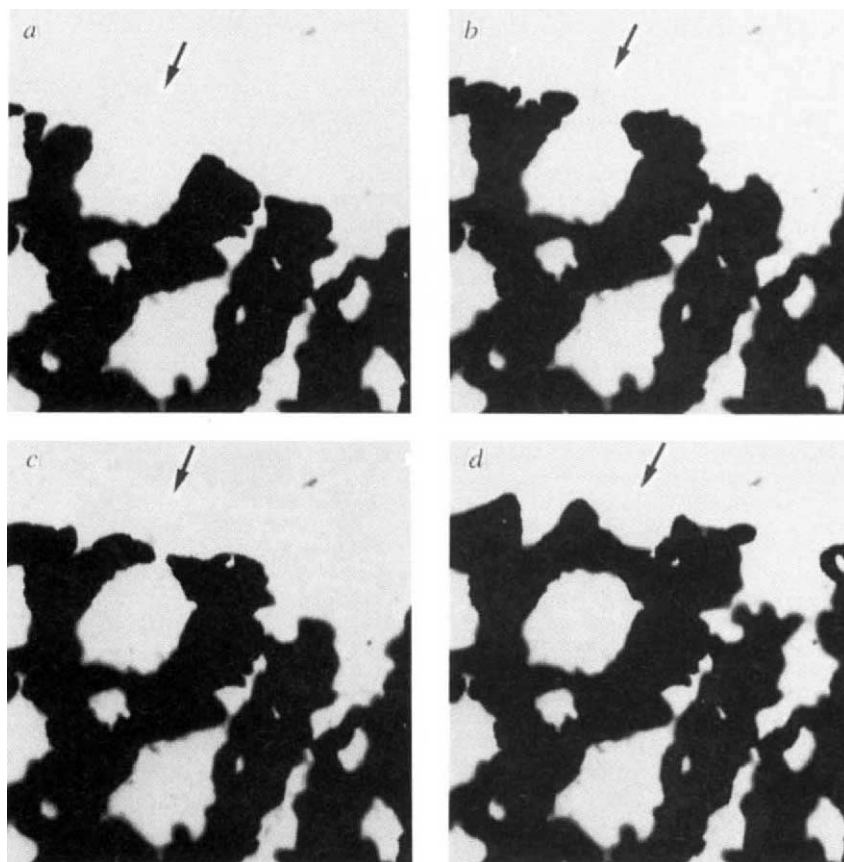


FIG. 2 The netting process during iron electro-deposition (experimental conditions as Fig. 1). As indicated by the arrow, previously separated tips of the deposit branches approach each other (a, b). When the two tips meet, a circular mesh is formed (c), and new branches are generated on the edge of the mesh (d). The process goes on, producing a network pattern. The time interval between two successive pictures is ~ 7 s. The thickness of the electrolyte solution film shown here is 70 μm . The net pattern and its mesh size do not change greatly when the thickness of the electrolyte film is reduced from 120 to 10 μm indicating that the netting phenomenon is not dependent on the geometry of the cell.



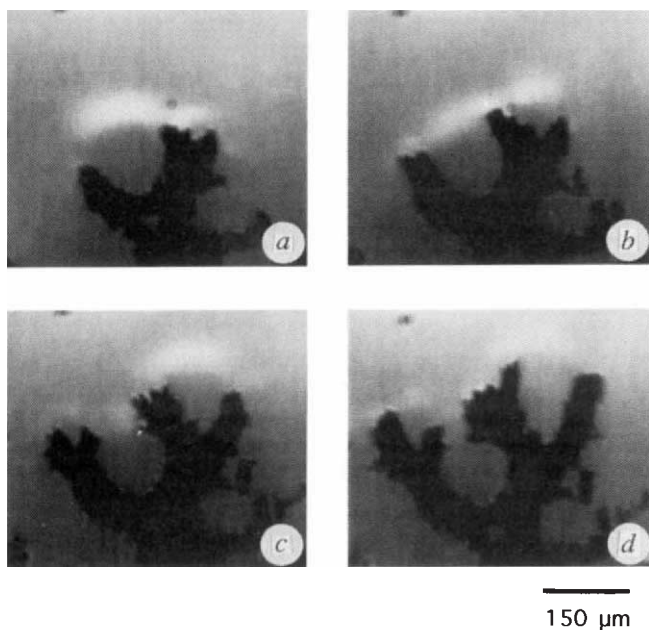
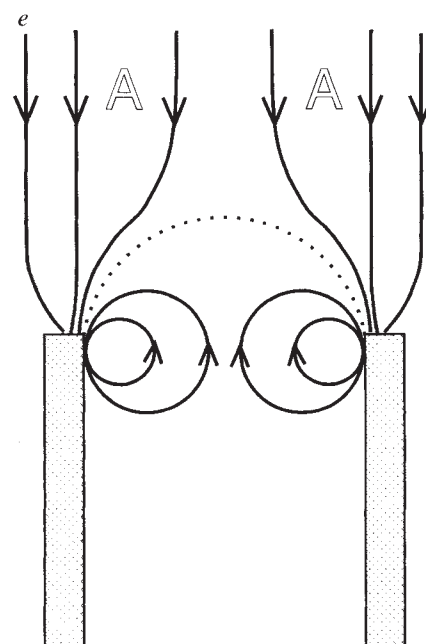


FIG. 3 a-d, Interference contrast micrographs of the concentration gradient in front of the growing tips during iron deposition, (experimental conditions as Fig. 1) showing bright arches bridging the neighbouring growing tips of the branches. As the deposit grows, the branches meet each other along the bright arch, the curvature and the contrast of which decreases as the branches approach each other. The arch finally disappears when the tips meet or become very close (c and d). e, Schematic diagram of the stream lines around two neighbouring deposit branches. The dashed line represents the virtual boundary between a zone free of ions (the area below the dashed line) and a zone with constant concentration (above the dashed line). The bright arch shown in a reflects the concentration gradient across this virtual boundary. Below the dashed line, the stream lines have the shape of vortices

bouring branches in Fig. 3a-d reflect the concentration gradient across the extended virtual boundary. However, as soon as Fe^{2+} diffused across the boundary, the existing vortices in the depleted region will transport Fe^{2+} ions to the facing parts of the neighbouring tips (Fig. 3e). The stream lines of the vortices are converging to the tips of the deposit, so the region on the tip which is facing the neighbouring one has more chance to grow. Consequently, the neighbouring tips tend to approach each other along the arch (Fig. 3). When two tips meet, a 'mesh' is formed. There-

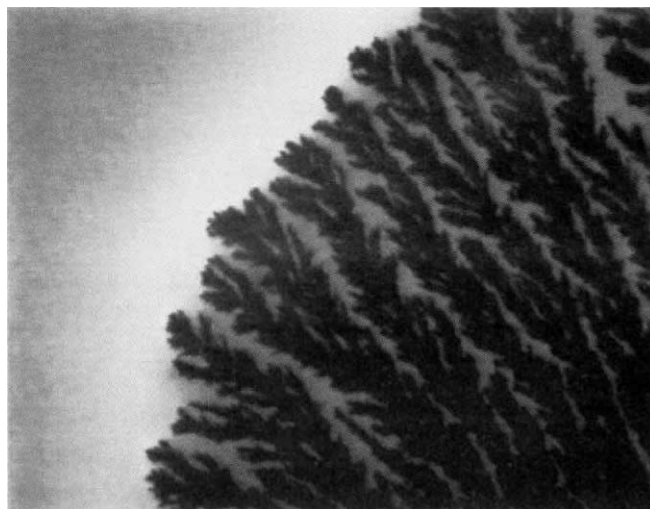
FIG. 4 After continued growth of the 'net-pattern' iron deposit shown in Fig. 1 (typically several millimetres in length), the deposit changes to a dense branching morphology (DBM), shown here as an interference contrast micrograph. There are no bright arches connecting the growing tips and the deposit branches no longer approach each other. Regions of solution with higher contrast (in front of the growing DBM interface) can be seen, showing a concentration gradient in the diffusion field. The concentration field is more uniform now and forms an envelope around the growing pattern. When the growth morphology changes to DBM, the number of the small diffusing particles increases gradually. The particles trapped between the deposit branches do not circulate, which is different from the situation in the earlier net pattern growth (Fig. 3). The pH of the electrolyte solution was found to have increased to ~ 4.0 after the morphology of the deposit changed to DBM. The time when the morphology changes depends on the initial H_3O^+ concentration and volume of the electrolyte solution, that is, the lower the initial pH and the thicker the aqueous film, the longer the net pattern will grow. The increase of pH is due to hydrogen generation and the consumption of H^+ ions. These experiments indicate that the interbranch convection depends on the H_3O^+ concentration, and the interbranch convection governs the morphology of the deposit.



(see text). Transmission optical dark-field microscopy (which can image particles down to $0.1 \mu\text{m}$ diameter in solution) and interference contrast microscopy are used to visualize the stream pattern by tracing the movement of small particles (possibly $\text{Fe}(\text{OH})_2$ or $\text{Fe}(\text{OH})_3$)¹⁴ during electrodeposition. During the netting process, the particles are found moving towards the branch tips along the streamlines A; on approaching the tips the velocity of the particles increases considerably. The particles trapped in the depleted area are found to move in circles (see text). The convection and net formation are independent of the solution film thickness and occur also if FeCl_3 instead of FeSO_4 is used. Therefore these phenomena are not caused by gravity-driven convection and anion chemistry.

after, under the influence of the diffusion field and the electric field, new branches will be generated on the edge of the mesh (Fig. 2d) and the process described above will repeat. In this way a mesh-like pattern is formed.

For convection to be initiated, a local force acting on the ions in the solution in the vicinity of the tip of the deposit branches is required. Fleury *et al.*⁹ have suggested that a small excess of metal ions near the tips of deposit branches provide a Coulomb force in an electric field which initiates convective motion. But



when deposit growth reaches a stationary state, a depleted zone is expected in front of the growing front, that is, the concentration of metal ion near the growing interface is lower. We therefore suppose that the contribution of the metal ions is small. Instead, we suggest that H_3O^+ ions accumulated around the growing front in our acidified solution are predominantly responsible for convection: during electrodeposition, H_3O^+ plays the role of an impurity and gradually accumulates in front of the growing interface while Fe^{2+} deposits on the cathode. When the concentration of H_3O^+ is sufficiently high, however, hydrogen bubbles will be nucleated, as indeed we observe in our experiments. So the overall H_3O^+ concentration in electrolyte solution decreases gradually as the electrodeposition goes on. Once the H_3O^+ concentration has decreased sufficiently, convection will be suppressed. This attenuation of convection is then responsible for the change of deposit morphology (Fig. 4).

We monitored the electric current during electrodeposition. The current fluctuates greatly and declines slightly during the growth of a mesh-like pattern, whereas it is steady and increases gradually for the DBM growth. The large fluctuation of the electric current may be attributed to the interbranch convection (M.W. *et al.*, manuscript in preparation). There is no great difference in electric current before and after the morphology transition. This may imply that a possible passivation of the anode and/or cathode by increasing the pH of the electrolyte solution does not affect the observed morphology transition. The role of H_3O^+ in stimulating interbranch convection is further supported by electrolysis of a pure H_2SO_4 solution film (pH ~ 2), with a graphite anode and inert branched metal needles as the cathode. It is found that if the applied voltage exceeds ~ 3 V, interbranch convection can be detected, either by directly monitoring the movement of intentionally added small particles near the cathodes or by measuring fluctuation of the electric current (M.W. *et al.*, manuscript in preparation).

It was reported recently that Marangoni-like (concentration-gradient-induced) effects on the surface tension at a liquid-liquid interface between the diffusion layer around the tip and the outer region may induce a convective instability even when the interface is not very sharp¹². In our case, the interfacial tension acting on the boundary separating the regions with different H_3O^+ and Fe^{2+} concentrations may provide a force that is mathematically equivalent to the electric force proposed in ref. 9. But the relative effects of interfacial tension and electric force cannot be distinguished in our experiments. However, the observed convection with stream lines shown in Fig. 3e indicates that some force acts, whatever its origin. Our experiments indicate that the formation of the mesh pattern is closely related to the presence of convection. □

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Self-assembly of a double-helical complex of sodium

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SPONTANEOUS self-organization of helical and multiple-helical molecular structures occurs on several levels in living organisms. Key examples are α -helical polypeptides, double-helical nucleic acids and helical protein structures, including F-actin, microtubules and the protein sheath of the tobacco mosaic virus¹. Although the self-assembly of double-helical transition-metal complexes^{1–15} bears some resemblance to the molecular organization of double-stranded DNA, selection between monohelical, double-helical and triple-helical structures is determined largely by the size and geometrical preference of the tightly bound metal¹⁴. Here we present an example of double-helical assembly induced by the weaker and non-directional interactions of an alkali-metal ion with an organic ligand that is pre-organized into a coil. We have characterized the resulting complex by two-dimensional NMR and fast-atom-bombardment mass spectrometry. These results provide a step toward the creation of molecular tubes or ion channels consisting of intertwined coils.

Previous strategies for self-assembly of helical metal complexes generally employed two types of polydentate ligands as metal-binding strands: bidentate or tridentate pyridine-type binding sites connected by relatively flexible links^{6,8,11–13,15–18}, or oligopyridines containing four to six pyridine rings^{7,10,14,19}. We have explored the coordination chemistry of molecular coils **1**

and **2** (Fig. 1). These ligands, also termed expanded heterohelicenes²⁰, are even less flexible than the oligopyridines. In all the previously studied cases of double-helical self-assembly, the ligands are conformationally reorganized by strong coordination of transition-metal or lanthanide ions. This process is shown in Fig. 1 for assembly of a monohelical 1:1 complex of silver with 2,2':6',2'':6'',2''':6''',2''''-quinquepyridine (**3**), which also forms double-helical 2:2 complexes of smaller metals preferring tetrahedral or octahedral coordination geometries. The double-helical complexes of unsubstituted oligopyridines^{14,19} and substituted analogues (for example quaterpyridine **4**, Fig. 1 and ref. 4) show discontinuities in the helical twist of each strand, which have planar metal-binding regions and twisted segments connecting these sites.

The difference between molecular coils **1** and **2** (Fig. 1) and the oligopyridines is that in **1** and **2** all adjacent pyridine rings are linked by an ethylene bridge (CH_2CH_2). The preference of each ethylene bridge for a staggered conformation introduces a twist between adjacent pyridine rings, as shown in Fig. 2a. This twist, torsional angle ϕ , falls in the range of 2–27° in the solid-state structures of comparable molecules^{21–23}. Torsional angles $<10^\circ$ have been observed only in a stacked 2:2 complex of **5**, a torand (Fig. 1), with lithium picrate²³; in the absence of crowding, 3,3'-ethylene-bridged 2,2'-bipyridines should assume the 18° N–C–C–N torsional angle obtained from force-field calculations²⁴. Pyridine has a large dipole moment (2.2 D), and electrostatic repulsion between adjacent dipoles should produce torsional angles of the same sign at each twist in the strand. Hence, molecular coils **1** and **2** have pre-organized helical shapes and form continuous helical bands, as shown in Fig. 2b. The geometric properties of this helical band, the radius (r equals the distance of each N atom from the helical axis) and the pitch (p), are determined by torsional angle ϕ . An increase in ϕ

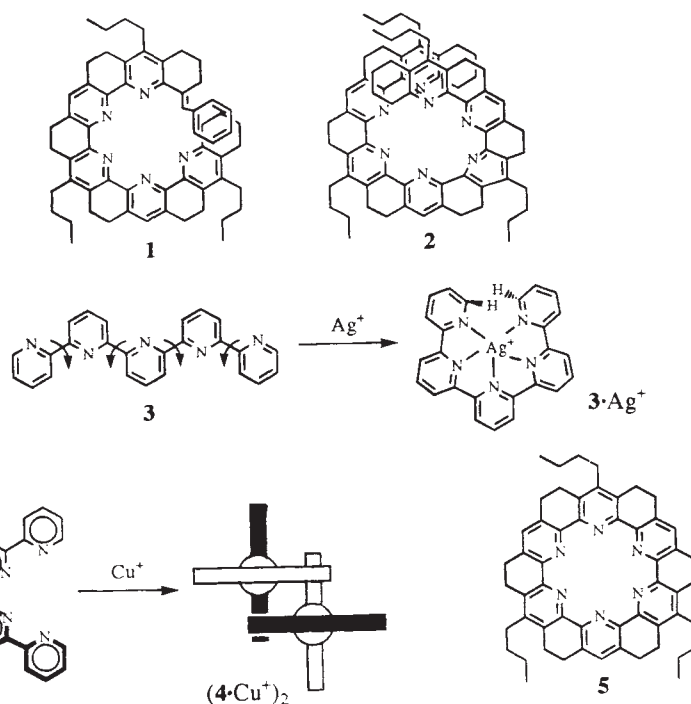


FIG. 1 Structures of expanded heterohelices (**1** and **2**), oligopyridines (**3** and **4**) and torand **5**. Systematic name of **1**: 3,9,23-tributyl-1,2,4,5,7,8,10,11,12,13,19,20,21,22,24,25-hexadecahydro-13(phenyl-methylene)dibenz-[j,j']acridino[4,3-b:5,6-b']di[1,10]phenanthroline. The perpendicular planes of each 5',3"-dimethyl-2,2':6',2'':6'',2'''-quaterpyridine ligand (**4**) are shown schematically in (**4**·Cu⁺)₂.

causes an increase in p and a decrease in r , enhancing the binding of smaller metal cations.

Compounds **1** and **2** apparently exist in solution as equal mixtures of right- and left-handed helices. Whereas the ethylene fragments in these compounds induce each twist between adjacent pyridines, the ring-inversion energy barriers are expected to be very low²⁴ enabling rapid equilibration between the right-handed and left-handed helical forms (enantiomers). The hydrogen atoms of every CH₂ group of a helix become axial (H_a) or equatorial (H_e), as shown in Fig. 2a; hence they are non-equivalent and should produce ¹H NMR resonances at different frequencies. The ¹H NMR spectrum of **1** (Fig. 4a) displays sharp signals, indicating that helix inversion occurs rapidly (>100 s⁻¹) at room temperature.

Molecular coil **1** binds Na⁺ to form double-helical 2:1 and 2:2 complexes, as shown in Fig. 3. Reaction of **1** with sodium trifluoromethanesulphonate (Na⁺CF₃SO₃⁻, NaOTf) in chloroform (reaction B) gave a complex having empirical formula 1(NaOTf)_{0.5}CHCl₃. When a methanol solution of **1** was boiled

with NaOTf (reaction A), a complex of composition 1·NaOTf·H₂O resulted. When CDCl₃ solutions of 1(NaOTf)_{0.5}CHCl₃ are allowed to stand in the dark (reaction C), two sets of ¹H NMR signals appear in 1:1 ratio, corresponding to 1·NaOTf·H₂O and the protonated ligand (1·H⁺). This process is faster in ambient light, probably due to generation of acid by photochemical decomposition of CDCl₃. The mass spectrum of 1·NaOTf·H₂O indicates that this is actually a 2:2 complex (1₂(NaOTf)₂). By analogy, 1(NaOTf)_{0.5}CHCl₃ would exist in solution as a 2:1 complex 1₂·NaOTf. Structurally related torand **5** forms strong 1:1 complexes with alkali metals, but no 2:1 complexes have been isolated^{25,26}. Despite the planar structure of **5**, which would favour face-to-face association, 2:1 and 2:2 complexes of **5** with alkali metals are unstable in solution. This suggests that 1₂·Na₂⁺ and 1·Na₂⁺ do not have sandwich structures; their stabilities can be explained by intertwining of the two molecular coils.

Figure 4 displays the 600 MHz ¹H NMR spectra of CDCl₃ solutions of **1** and 1₂·Na₂⁺; the assignment of the peaks and the

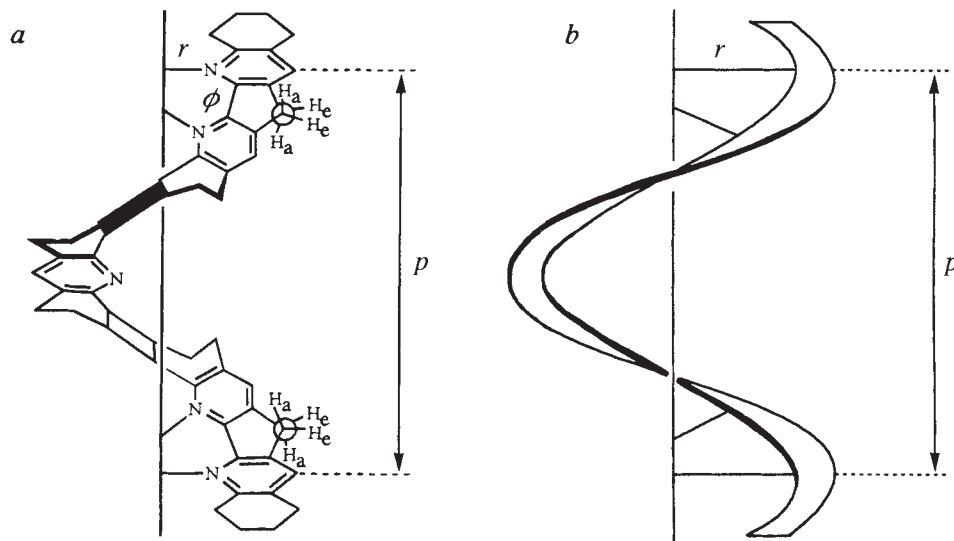
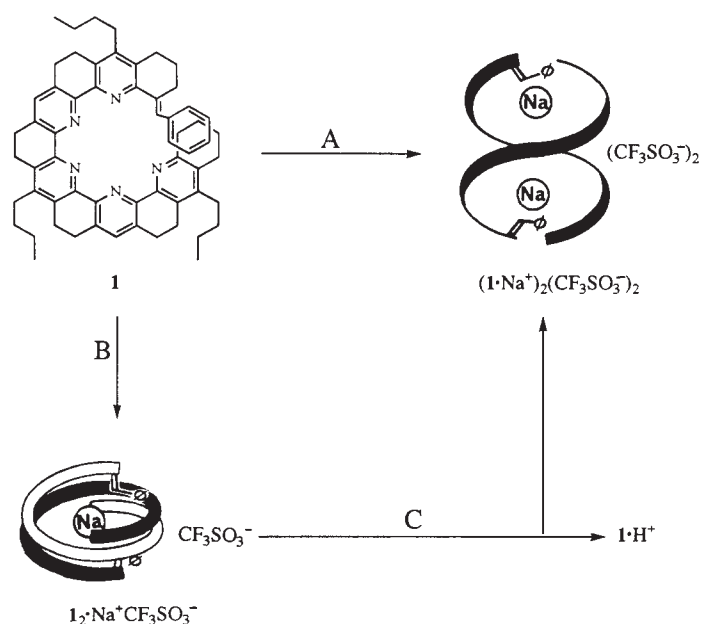


FIG. 2 Schematic diagrams showing proposed helical shape of **2**. a, Helical conformation resulting from positive N-C-C-N torsional angles (ϕ) at each 2,2'-bipyridine linkage, showing helical radius (r), pitch (p) and orientations of axial (H_a) and equatorial (H_e) hydrogen atoms of two ethylene bridges linking 3 and 3' positions of 2,2'-bipyridine fragments (butyl groups omitted for clarity); b, Schematic representation of **2** as a continuous helical band.

FIG. 3 Synthesis of $\text{1}_2(\text{NaOTf})_2$ and $\text{1}_2 \cdot \text{NaOTf}$.

METHODS. Reactions were conducted under an atmosphere of anhydrous N_2 as follows. Reaction A; a solution of 226 mg (0.3 mmol) of **1**¹⁹ in 10 ml of methanol was stirred with 46 mg (0.3 mmol) of sodium trifluoromethanesulphonate (NaOTf) (64 °C, 6 h). The solvent was evaporated, 10 ml of CH_2Cl_2 was added and the resulting mixture was filtered. The filtrate was chromatographed with basic alumina ($\text{CH}_2\text{Cl}_2/\text{isopropanol}$, 9:1) and the residue from the purest fraction was dissolved in 0.5 ml of CH_2Cl_2 . Addition of this solution to 30 ml of ether (0 °C) gave a beige precipitate, which was isolated by filtration and dried *in vacuo* (60 °C) yielding 50 mg (18%) of $\text{1} \cdot \text{NaOTf} \cdot \text{H}_2\text{O}$. Fast-atom-bombardment mass spectrometry (FAB-MS) of this compound gave peaks at mass/charge ratios (m/z) as follows: 1,756 AMU ($\text{1}_2 \cdot \text{Na}_2^+$); 878 AMU ($\text{1} \cdot \text{Na}^+$). Reaction B; a solution of 50 mg (0.06 mmol) of **1** in 1 ml of acid-free CHCl_3 was stirred with 10 mg (0.06 mmol) of NaOTf (0 °C, 1 h, dark). The CHCl_3 solution was filtered and the filtrate was evaporated under a flow of nitrogen in the dark. The resulting solid was dried *in vacuo* (60 °C) to give $\text{1}(\text{NaOTf})_{0.5} \cdot \text{CHCl}_3$. FAB-MS; m/z 1,733 AMU ($\text{1}_2 \cdot \text{Na}^+$). Reaction C; a CDCl_3 solution of $\text{1}_2 \cdot \text{NaOTf}$ was allowed to stand in the dark at 0 °C and gave $\text{1}_2(\text{NaOTf})_2$ and $\text{1} \cdot \text{H}^+$. Compositions of the products of reactions A and B were determined by combustion microanalysis (C, H, N, Na).

position of the protons in the molecules are also shown. Whereas the CH_2 signals (b–k) of the free ligand are sharp (Fig. 4A), the spectrum of $\text{1}_2 \cdot \text{Na}_2^+$ (Fig. 4B) shows several one-proton multiplets for ethylene bridge hydrogens (k, 2.0–3.3 p.p.m.). This spectrum was observed both for samples of $\text{1}_2(\text{NaOTf})_2$ and for samples of **1** which acquired Na^+ upon chromatography²⁰. Each pair of CH_2 protons becomes diastereotopic in the 2:2 complex because the helices no longer invert rapidly. The large upfield shifts observed in Fig. 4B, especially for H_0 (1.2 p.p.m.) and H_z (2.0 p.p.m.), are consistent with a double-helical structure in which the benzyldiene groups lie above, and are shielded by, pyridine rings of the other strand. Similar shielding effects have been reported by Lehn *et al.*^{6,8} and by Constable¹⁴ for double-helical complexes of polydentate pyridine ligands with Cu^+ and Cd^{2+} , respectively. The 300 MHz ^1H NMR spectrum (not shown) of $\text{1}_2 \cdot \text{NaOTf}$ in CDCl_3 also shows broadening of the CH_2 signals and upfield shifts relative to **1** (0.8 p.p.m. for H_0 and 1.5 p.p.m. for H_z).

By means of various two-dimensional NMR experiments (Fig. 5) axial and equatorial protons could be identified in each of the ethylene bridges, which were numbered C(1)–C(2), C(3)–C(4), C(5)–C(6) and C(7)–C(8). Interactions were seen in the ROESY (rotating frame Overhauser enhancement spectroscopy) spectrum for equatorial protons on carbons 1 and 4 (1e, 4e) with the most downfield pyridine proton (py_1), and for the equatorial protons on carbons 5 and 8 (5e, 8e) with the higher field pyridine proton (py_2 , Fig. 5B). These results permitted assignment of two fragments: C(3)–C(4)– py_1 –C(1)–C(2) and C(6)–C(5)– py_2 –C(8)–C(7). Besides interacting with neighbouring equatorial protons on the ethylene bridges, pyridine proton py_2 is near proton 5a and a methylene group of a terminal cyclohexane ring (or a proton of a butyl chain). Also, pyridine proton py_1 interacts with a methylene proton of a terminal cyclohexane ring or a proton of a butyl chain (c) or proton 2a, and H_z is near hydrogens (h) on both faces of the other terminal cyclohexane ring. These through-space interactions cannot be explained by a face-to-face structural model in which each ligand encircles a cation. Figure 5C shows through-space interactions between the two molecules of **1** in $\text{1}_2 \cdot \text{Na}_2^+$, and the proposed structural relationship between the molecular fragments within each molecule of **1**.

The spectroscopic results and CPK (Corey–Pauling–Koltun) models show that $\text{1}_2 \cdot \text{Na}_2^+$ has a structure in which both sodium ions are shared equally by two intertwined ligands. Moreover, the mechanical relationship between helical radius and pitch

(Fig. 2) explains why **1** forms a double-helical complex with Na^+ . As the radius of **1** at a minimum pitch would be similar to that of torand **5**, which matches potassium²², coiling of two molecules of **1** around two smaller ions should enhance metal–ligand interaction by decreasing the radius.

Compound **1** is the first molecule known to form double-helical complexes with alkali-metal ions. Binding of alkali metals

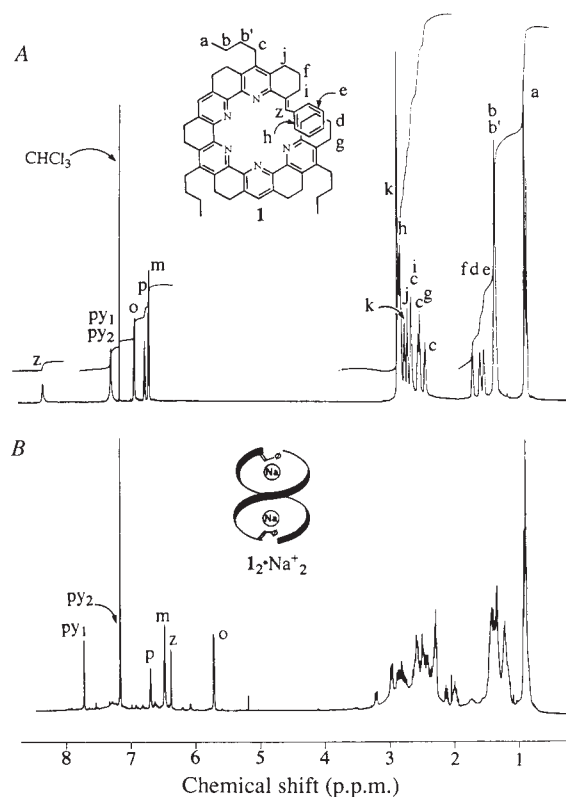


FIG. 4 ^1H NMR spectra (600 MHz, 293 K) of solutions of **1**(A) and $\text{1}_2 \cdot \text{Na}_2^+$ (B) in CDCl_3 . Peak assignments are indicated by labels in the diagram of **1**, except: ethylene bridges (k), pyridines (py_1 , py_2) and benzene *ortho*-*meta*- and *para*-protons (o, m and p, respectively). NMR samples of $\text{1}_2 \cdot \text{Na}_2^+$ were obtained by careful chromatography of crude **1**¹⁹ with alumina and contained 8–10% impurity of $\text{1}_2 \cdot \text{Na}^+$. (Chemical shift is p.p.m. with respect to CHCl_3 , 7.26 p.p.m.).

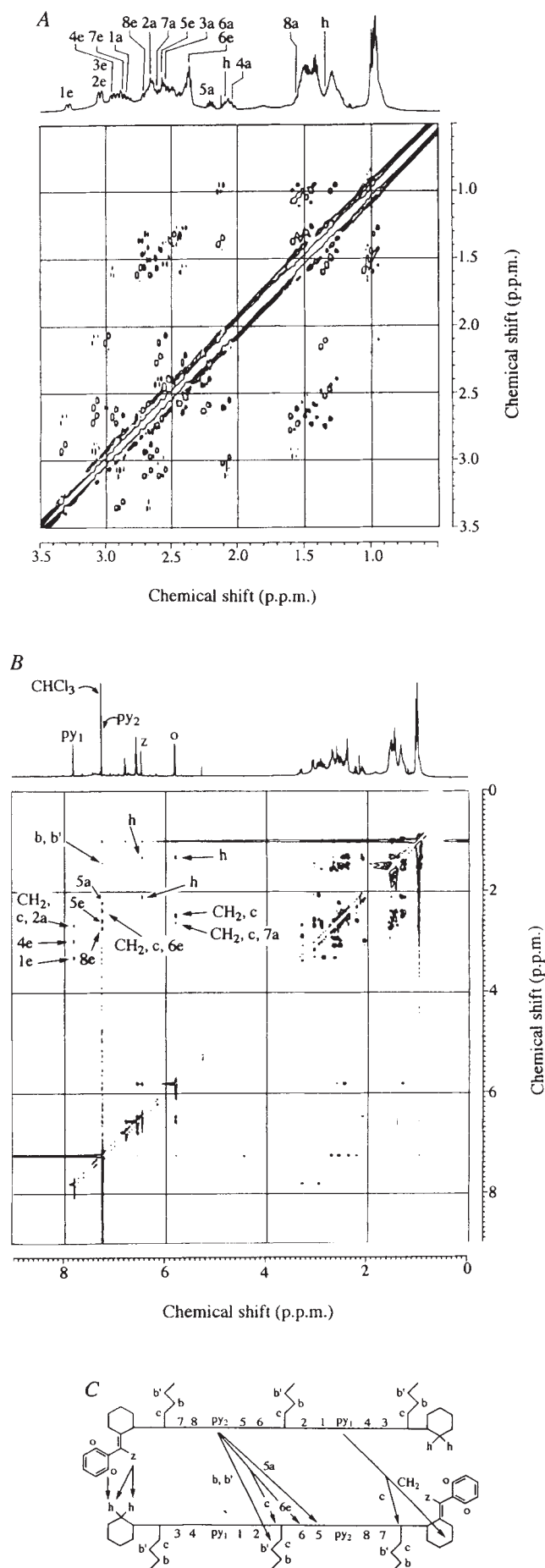


FIG. 5 A, ^1H NMR (600 MHz, CDCl_3 , 293 K) and double-quantum-filtered, phase-sensitive correlated spectroscopy (DQF-PS COSY) spectra (0.5–3.5 p.p.m.) of $\mathbf{1} \cdot \text{Na}_2^+$ (ethylene bridge protons assigned to carbons 1–8 with letters a or e indicating axial or equatorial orientation; compare Fig. 2a), B, ROESY spectrum of $\mathbf{1} \cdot \text{Na}_2^+$ (cross peaks marked by arrows indicating through-space interactions between protons py_1 , py_2 , z or o and higher field protons). C, Schematic representation of a proposed structure displaying through-space interactions between protons of the two molecules of $\mathbf{1}$ in $\mathbf{1} \cdot \text{Na}_2^+$.

METHODS. Ethylene spin systems were identified by means of the DQF-PS COSY spectrum shown and a ^1H - ^{13}C one-bond correlation spectrum (not shown). Axial and equatorial protons were assigned according to observed geminal and vicinal ^1H - ^1H coupling constants. Ethylene-bridge carbon atoms adjacent to 4-substituted pyridine rings were assigned by means of ^{13}C NMR chemical shifts.

by pyridine ligands is weaker than coordination of pyridines to transition metals or lanthanides²⁷, responsible for previously discovered self-assembling helicates. Induction of self-assembly by relatively weak metal–ligand forces suggests that $\mathbf{1}$ may also undergo dynamic exchange between monomeric and double-helical forms in solution before metal coordination. Double-helical aggregation of longer molecular coils might occur without metal complexation. The resulting tubular structures would resemble the proposed double-helical form of gramicidin²⁸, a channel-forming antibiotic, and might mimic the ion transport of such self-assembling ion channels in lipid bilayer membranes. Eventually, structurally programmed supramolecular assemblies of this type may enable fabrication of nanoscale electronic and ionic devices^{29,30}. □

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Reassessing the dependence of cloud condensation nucleus concentration on formation rate

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MARINE stratocumulus clouds play an important role in the Earth's radiation budget. The albedo of these clouds depends on the cloud droplet size distribution, and therefore, in part, on the number density of cloud condensation nuclei (CCN)¹. It has been postulated² that a positive feedback loop between increased CCN concentrations and decreased drizzle gives rise to a bistable system, in which there are two equilibrium CCN concentration regimes. According to this model, CCN concentration is only weakly dependent on the CCN production rate within the stable regimes, but very strongly dependent on this rate in the transition region between the regimes. If correct, this strong dependence implies that a small increase in the production of CCN over the oceans could drastically increase the planetary albedo. Using a more sophisticated model³ than that used previously, we find no evidence for bistability. However, we find that CCN concentrations are generally strongly dependent on their production rate, so that changes in the latter would influence the Earth's albedo. We also find that the timescale for reducing high CCN concentrations can be as long as several days, which implies that high CCN concentrations can persist in clouds advected to regions of lower CCN production rate.

Baker and Charlson² (hereafter referred to as BC) suggested that the marine cloud-topped boundary layer is bistable with respect to CCN concentrations. When the production rate of CCN is low, the coalescence of droplets (which produces drizzle) maintains low CCN concentrations. Baker and Charlson identify this stable state with a clean marine boundary layer, with equilibrium CCN concentrations of $\sim 5\text{--}50\text{ cm}^{-3}$. At higher CCN concentrations in the BC model, cloud droplets were smaller and droplet coalescence was not efficient enough to balance the CCN production rate. A balance was not achieved until CCN concentrations reached $\sim 800\text{ cm}^{-3}$; above this concentration the coagulation of dry CCN balanced their production rate. Because the albedo of marine stratiform clouds is sensitive to cloud droplet concentrations, the bistability hypothesis of BC suggests that global climate could depend on the production rate of CCN over the oceans in a highly nonlinear fashion.

In view of the potential importance of the BC bistability hypothesis, we have re-evaluated the relationship between CCN concentration and production rate using a more sophisticated model³. Both our model and the BC model assume that CCN are produced at a constant rate by unspecified processes; in other respects, the two models are quite different.

In the BC model, all unactivated CCN are assumed to be the same size. When liquid water is present, all CCN are assumed to activate to form droplets. The droplet spectrum is separated into two size classes: cloud droplets and drizzle drops. The reduction of particle concentration by droplet coalescence is calculated using an empirical formula to describe the drizzle flux and a simplified relationship to describe the efficiency with which drizzle drops collect cloud droplets. The cloud-microphysics component of our model⁴ resolves the size distributions of both unactivated CCN and cloud droplets into 50 size classes, ranging in radius from 0.005 to $500\text{ }\mu\text{m}$. Also, it treats explicitly the physical processes of CCN activation, droplet condensational growth and evaporation, the aggregation of particles due to thermal coagulation and the coalescence of particles due to gravita-

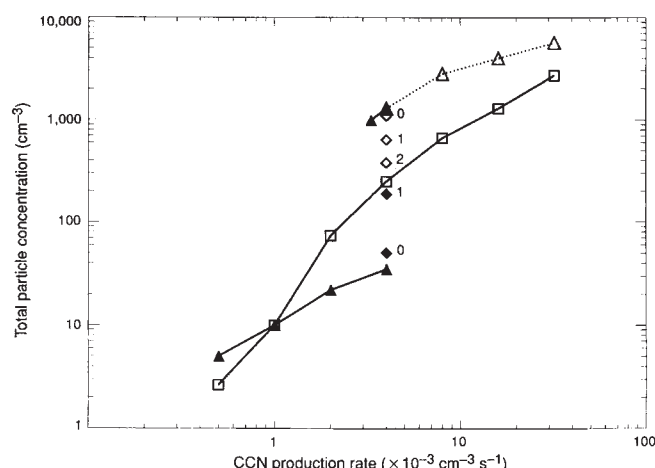


FIG. 1 Variation of equilibrium total particle concentration (N) averaged over the depth of the boundary layer, with CCN production rate (S_0). Squares connected by a solid line are results from the present model. Solid triangles connected by a solid line are results from the model of Baker and Charlson². Open triangles connected by a dotted line represent a balance between the CCN source and CCN sink by dry particle coagulation, using the formulation of Baker and Charlson. Also shown are the progressions toward equilibrium values of N from the present model simulations, starting with total concentrations of $1,100\text{ cm}^{-3}$ (open diamonds) and 50 cm^{-3} (solid diamonds); the number alongside each point is the number of days elapsed after initialization. N reached the same equilibrium value after 2 and 3 days for initial values of 50 and $1,100\text{ cm}^{-3}$, respectively.

tional collection. The single-layer model of BC considers the boundary layer to be well mixed and to have a prescribed depth. Our model resolves the boundary layer into ~ 40 layers, and treats turbulent transfer between layers⁵; boundary layer depth is determined by a dynamic balance between subsidence and turbulent mixing. The turbulent mixing in the BC model is driven by drizzle and a fixed net radiative flux loss from the boundary layer. Our model employs a sophisticated radiative transfer scheme⁶ that is coupled to the microphysical and thermodynamic fields. Baker and Charlson did not address time evolution issues because their steady-state model does not treat the dynamics between mixing, radiative transfer and cloud microphysics. Our model treats the evolution of the coupled system.

Although our model of the marine boundary layer is more detailed than the BC model, it has some shortcomings. Only one spatial dimension (the vertical) is represented. Therefore, horizontal heterogeneity and horizontal advection are ignored. Our treatment of vertical mixing assumes all turbulent transfer is down-gradient, which is not always the case. Only mean quantities are predicted by our model—variances are not treated. Despite these deficiencies, our model reproduces many of the observed characteristics of the cloud-topped marine boundary layer³. Model results compare well with measurements of stratiform cloud layers in the North Sea^{7,8}. In these comparisons, the model was able to reproduce measured variations (including drizzle rates) between clouds observed under different conditions.

To evaluate the sensitivity of CCN concentrations to CCN production, we ran our model with fixed boundary conditions to steady state (within the variation of diurnal oscillations). The only change between model runs was the CCN production rate (S_0), which we prescribed through the depth of the boundary layer using a log-normal aerosol distribution with a mean (by number) geometric radius of $0.1\text{ }\mu\text{m}$. For all our simulations, the model domain was initially cloudless and subsaturated with respect to water. The initial temperature lapse rate was adiabatic up to an inversion height of 750 m , where there were jumps in temperature ($+6\text{ K}$) and water vapour mixing ratio (-2 g kg^{-1}).

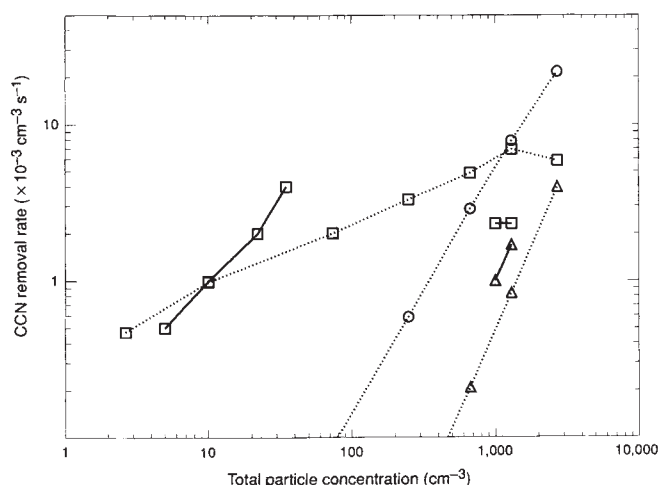


FIG. 2 Sink terms in the budgets for average total particle number concentrations (N) as a function of N . Dotted lines are from the present model, solid lines are from model of Baker and Charlson². In both cases, squares represent removal due to drizzle (by coalescence of droplets and deposition of drops on the ocean), and triangles represent particle removal due to coagulation between dry CCN. Collection of dry CCN particles by droplets in the present model is indicated by open circles.

At the lower boundary of the model, the sea surface temperature was fixed at 288 K. The downwelling infrared flux at the top of the model (1 km) was fixed at 270 W m^{-2} , and the wind speed in the top layer of the model was fixed at 8.5 m s^{-1} . The model simulations were started at midnight, with the sun rising four hours later and climbing to reach 35° from zenith, at noon. Over the long times required to reach steady-state conditions, the upward entrainment of the boundary layer was limited by a constant divergence rate of $5 \times 10^{-6} \text{ s}^{-1}$. Particles above the boundary layer were not advected downward by subsidence.

A comparison of our results with those of BC is shown in Fig. 1, where the total particle concentration (N) is the sum of the concentrations of unactivated CCN and cloud droplets averaged over the depth of the boundary layer. In this letter, N is referred to as the "CCN concentration". We have extended BC's results beyond the maximum source strength that they presented ($S_0 = 0.004 \text{ cm}^{-3} \text{ s}^{-1}$) by balancing the CCN source with the dry coagulation sink used in their model. Although the end points of the relationships from the two models are in agreement, the intermediate behaviour is very different. In contrast to the discontinuous jump in N at $S_0 \approx 0.004 \text{ cm}^{-3} \text{ s}^{-1}$ that the BC formulation gives, our model produces a smooth dependence of N on S_0 . The BC relationship between N and S_0 is nearly linear in both regimes. In our results the strongest dependency (nearly cubic) occurs in the region where N increases from 10 to 74 cm^{-3} in response to a doubling of S_0 from $0.001 \text{ cm}^{-3} \text{ s}^{-1}$. This peak in the N - S_0 dependence occurs in a regime where the effects of changes in CCN concentrations on radiatively driven turbulent mixing are greatest⁹.

The reasons for the differences between our results and those of BC are evident from the CCN removal rates shown in Fig. 2. In the BC model there are three sinks for CCN; dry particle coagulation, coalescence of cloud droplets with drizzle drops and deposition of drizzle drops to the ocean. We have combined the last two sinks and presented them as the removal rate of CCN by drizzle (only at the lowest CCN concentrations is droplet deposition significant). To understand our results, it is important to distinguish between the drizzle flux and the removal of droplets by coalescence, because they are not simply related. For instance, when N rises from 10 to $1,300 \text{ cm}^{-3}$ (and droplet concentrations rise from 7 to 500 cm^{-3}), the drizzle flux reaching the surface in our model decreases by a factor of 12 because the size of the drizzle drops decreases, but the removal of droplets

through coalescence increases by a factor of 7 because the number of droplets increases (Fig. 2).

At CCN concentrations below $\sim 50 \text{ cm}^{-3}$, droplet coalescence increases more rapidly with N in the BC model than in our model (Fig. 2). Therefore, at low CCN concentrations, N is less dependent on the CCN production rate in the BC model than it is in our model (Fig. 1). Droplet coalescence peaks at a droplet concentration of $\sim 50 \text{ cm}^{-3}$ in the BC model (Fig. 2). At higher particle concentrations, a balance with the CCN source can only be restored in the BC model when coagulation of CCN becomes significant. This gap in CCN concentrations, between where droplet coalescence is able to balance the CCN source and where dry coagulation is fast enough to maintain equilibrium CCN concentration, is called the "drizzle pause" by BC. The gap exists in the BC model because their total removal rate (the sum of drizzle removal and dry coagulation) initially decreases for droplet radii $< 10 \mu\text{m}$, which corresponds in their simulations to CCN concentrations $> 50 \text{ cm}^{-3}$. In our model, a decrease in droplet coalescence does not occur until much higher CCN concentrations are reached, where another removal process, the collection of CCN by droplets, is significant. There is neither a "drizzle pause" nor a discontinuity in equilibrium total particle concentrations in our model because the total removal rate never decreases as N increases.

The steady-state response of marine clouds to changing CCN production rates will not be realized unless the adjustment times are shorter than other relevant timescales (for example, the periods for which steady boundary conditions and steady CCN production rates prevail). We have evaluated the timescales required for CCN concentrations in the boundary layer to reach their equilibrium concentrations by performing model simulations in which N was initialized above and below its equilibrium value, and then following progress towards equilibrium. For both simulations, a source strength of $0.004 \text{ cm}^{-3} \text{ s}^{-1}$ was used, which produces an equilibrium N of 250 cm^{-3} (Fig. 1). To approach equilibrium from above, N was initialized at $1,100 \text{ cm}^{-3}$; equilibrium from below was approached by initializing N at 50 cm^{-3} . The progress of these simulations toward equilibrium is depicted in Fig. 3, where it is seen to take three days to reach equilibrium from an initial value of $1,100 \text{ cm}^{-3}$, while two days are required for the simulation initialized at 50 cm^{-3} . These are long periods of time relative to the time available for a cloud-topped marine boundary layer to evolve under conditions of roughly constant CCN production and steady boundary

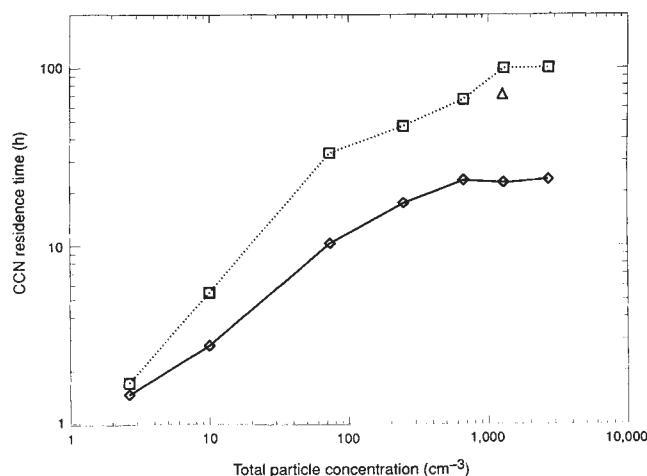


FIG. 3 Variations of CCN residence times (τ) with total particle number concentrations (N). Squares connected by the dotted line show residence time with respect to CCN mass concentration (τ_M). Diamonds connected by the solid line show residence time with respect to CCN number concentration (τ_N). Triangle is τ_M when the minimum deposition velocity is set to 0.1 cm s^{-1} .

conditions. For this reason, equilibrium CCN concentrations may often not be realized in nature. For example, marine boundary layer clouds that form in air with high CCN concentrations should retain high droplet concentrations for several days, provided that the microphysics of the cloud is determined by the CCN concentration rather than changing boundary conditions.

Residence times are another useful measure of CCN lifetimes. The residence time (τ) is equal to the equilibrium concentration of a quantity divided by its production rate. Using our results, the residence times for CCN number concentration (τ_N) and mass concentration (τ_M) are shown in Fig. 3. The three-day relaxation of N from an initial value of $1,100 \text{ cm}^{-3}$ to its equilibrium value of 250 cm^{-3} (Fig. 1) is consistent with an e-folding time of one day, which is close to the values shown in Fig. 3.

Because many processes that reduce CCN number concentrations do not affect CCN mass concentrations, τ_M always exceeds τ_N . As these two residence times can differ so much (Fig. 3), it is important to distinguish between them when estimating the effects of aerosols on cloud properties.

At low CCN concentrations, the deposition of drizzle on the ocean is the dominant sink for CCN mass, whereas the coalescence of cloud droplets is the dominant sink for CCN numbers. At high CCN concentrations, as the removal of CCN mass by drizzle reaching the ocean gradually becomes insignificant, τ_M is controlled by the dry deposition of CCN to the ocean. For CCN deposition velocities, we use the formulation of Giorgi¹⁰, in which the deposition velocity is a minimum ($\sim 0.01 \text{ cm s}^{-1}$) for particles of radius $\sim 0.1 \mu\text{m}$. Because CCN deposition velocities

are uncertain, we ran an additional simulation in which the minimum value of the deposition velocity was 0.1 cm s^{-1} . This reduced our calculated value of τ_M by $\sim 25\%$, as shown in Fig. 3, but had negligible effect on τ_N .

Although we find no evidence of a bistability in the steady-state relation between CCN concentrations (N) and source strength (S_0), we agree with BC that N is strongly dependent on S_0 . Accordingly, there is a significant potential for changes in CCN production rates to change cloud droplet size distributions, cloud albedo, and therefore, climate. We find, however, it can take several days for N to reach equilibrium values. Therefore, clouds should be more sensitive to the highest CCN concentrations to which they are exposed than to the CCN production rates in regions to which they may be advected. □

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Rapid interglacial climate fluctuations driven by North Atlantic ocean circulation

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RECENT data from the GRIP ice core^{1–3} in Greenland suggest that the climate of the last (Eemian) interglacial period was much less stable than that of the present interglacial. Rapid transitions between warm and cold periods were found to occur on timescales of just a few decades. The North Atlantic climate during the Eemian period was also shown to be characterized by three states, respectively warmer than, similar to and colder than today^{1,2}. Recent data from the nearby GISP2 ice core have revealed some discrepancies with these findings, which remain to be resolved^{4,5}. Here we present simulations using an idealized global ocean model, which suggest that the North Atlantic ocean has three distinct circulation modes, each of which corresponds to a distinct climate state. We find that adding a simple random component to the mean freshwater flux (which forces circulation) can induce rapid transitions between these three modes. We suggest that increased variability in the hydrological cycle associated with the warmer Eemian climate could have caused transition between these distinct modes in the North Atlantic circulation, which may in turn account for the apparent rapid variability of the Eemian climate.

One of the most important indicators of past climates and their variability is the record from the Greenland ice cores. Early analyses of these ice cores have shown that the climate of the North Atlantic during the last glaciation was marked by abrupt swings between relatively warm and relatively cold regimes^{6–8}. These climate shifts were observed to occur very rapidly (over several decades) and quite frequently (every several thousand

years or so). Similar oscillations have been found during the transition between the glacial and interglacial climates, the most recent of which was the Younger Dryas event which ended $\sim 10,000 \text{ yr ago}$ ^{9–11}. Although it has long been known that the climate of our present Holocene period has been marked by much weaker natural climate variability on both the century¹² and decadal¹³ timescales, we have not observed any rapid and dramatic climate shifts.

The Greenland Ice-core Project (GRIP) 3028.8-m ice core from Summit, Greenland^{1,2} provides high-resolution climatic information through the Eemian and into the penultimate glaciation. The Greenland Ice Sheet Project 2 (GISP2) recently reported results from a 3,053.4 m core drilled 28 km to the west of the GRIP core^{4,5}. Although the two cores correlated extremely well over the top 90% of the record, the correlation in the rest

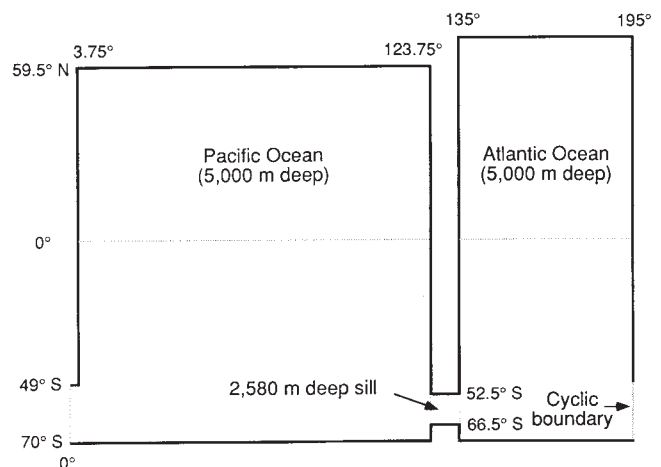


FIG. 1 The geometry (plan view) of the idealized two-basin model used in this study. All longitudes are measured relative to the western boundary of the model domain.

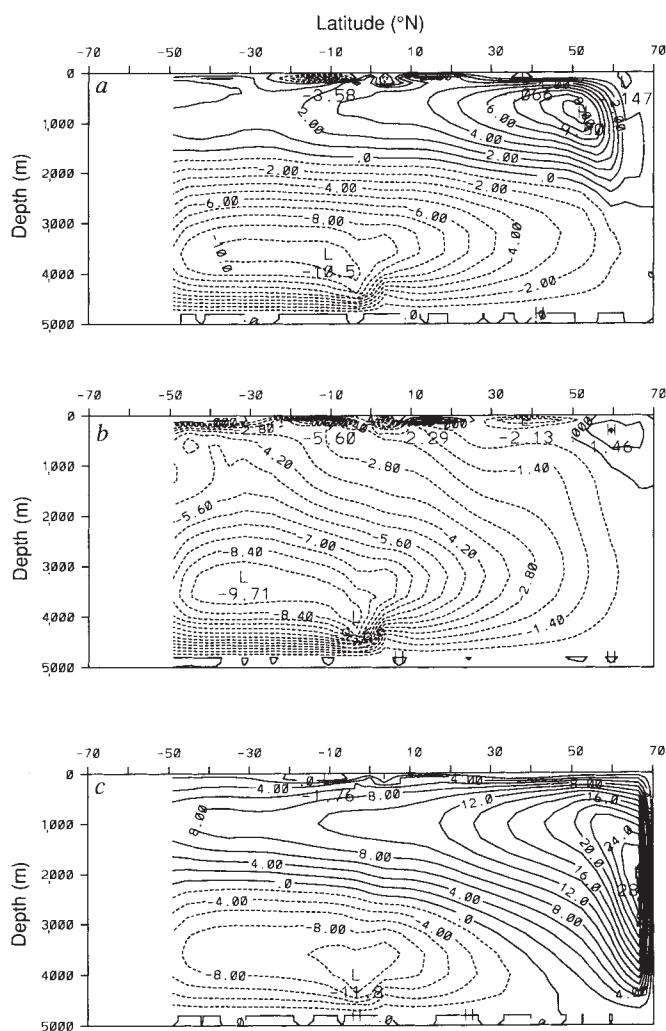


FIG. 2 The Atlantic Ocean meridional overturning streamfunction in Sv ($1 \text{ Sv is } 10^6 \text{ m}^3 \text{ s}^{-1}$) for the three equilibria. *a*, The normal 'present-day' conveyor. *b*, The weak 'colder' conveyor. *c*, The strong 'warmer' conveyor. The x-axis is the latitude with positive and negative values indicating °N and °S, respectively. Positive and negative contours indicate clockwise and counterclockwise circulations, respectively, with H and L indicating local maxima and minima, respectively.

of the core, including the Eemian, was poor⁵. Several reasons (most probably related to flow deformation at one or both of the core sites) for this poor correlation were discussed in refs 4 and 5. Nevertheless, it has been reported^{1,5} that over the first 2,847 m ($\sim 129,000$ yr) of the GRIP core, which includes most of the Eemian ($\sim 115,000$ – $135,000$ yr ago), there was no significant disturbance of the ice layering. Such disturbances were first observed at a depth of 2,678 m in the GISP2 core (well above the component of the core associated with the Eemian). Although the initial ice analysis suggests that "the GRIP record can be interpreted climatically to a greater depth than the GISP2 record"⁵, we must await more detailed ice-core analysis to confirm the GRIP findings. We suggest here that our model results are, however, consistent with the GRIP findings.

Previous theories of the glacial millennial-timescale oscillations have involved an interaction between continental ice sheets, the Atlantic-to-Pacific hydrological cycle and the thermohaline circulation of the North Atlantic¹⁴. It has further been proposed that the Younger Dryas event was just one such cycle modified by the division of melt water from the Mississippi to the St.

Lawrence¹⁵. These theories cannot apply to the Eemian period as the large-scale continental ice sheets were not in existence then. Here we suggest that the fluctuations of the ocean's thermohaline circulation can alone explain such rapid climate shifts.

We use an idealized (Fig. 1) ocean general circulation model (OGCM)¹⁶ forced by idealized steady winds¹⁷. For economy of calculation we use coarse horizontal resolution (3.75° meridional $\times$ 3.5° zonal) with 19 vertical levels varying from 50-m grid box thickness near the surface to 420-m grid box thickness near the bottom. The model was initially integrated for 3,288 yr to equilibrium by relaxing the surface temperatures and salinities of the ocean to observed zonally-averaged Pacific and Atlantic values¹⁸ with a timescale of 50 d.

On reaching the 'present-day' climatological equilibrium under the relaxation boundary conditions, the amount of fresh water into and out of each surface grid box was determined. This diagnosed flux was then used as a specified boundary condition on salinity, while the thermal boundary condition was left unchanged (known as mixed boundary conditions; see, for example, ref. 19) for all further integrations. In the absence of a fully prognostic atmospheric model, mixed boundary conditions are often used to reflect the fundamental difference in evaporation–precipitation and thermal coupling between the ocean and the atmosphere.

The 'present-day' climatology was perturbed many times in an attempt to determine all possible equilibria of the idealized system. This exhaustive procedure yielded only three different configurations for the North Atlantic conveyor²⁰. Figure 2 illus-

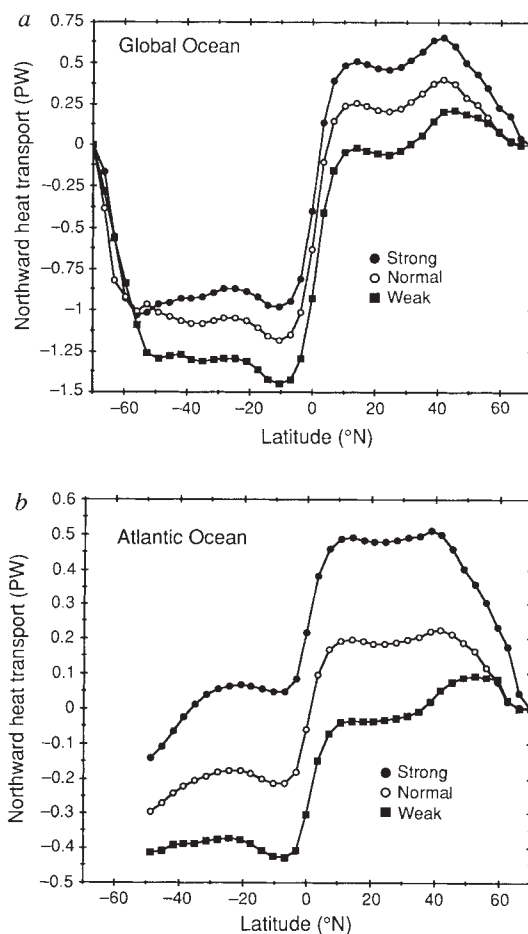


FIG. 3 The poleward heat transport in PW ($1 \text{ PW is } 10^{15} \text{ W}$) associated with the three equilibria portrayed in Fig. 2. *a*, The global ocean (Pacific + Atlantic + Southern oceans). *b*, The Atlantic Ocean only.

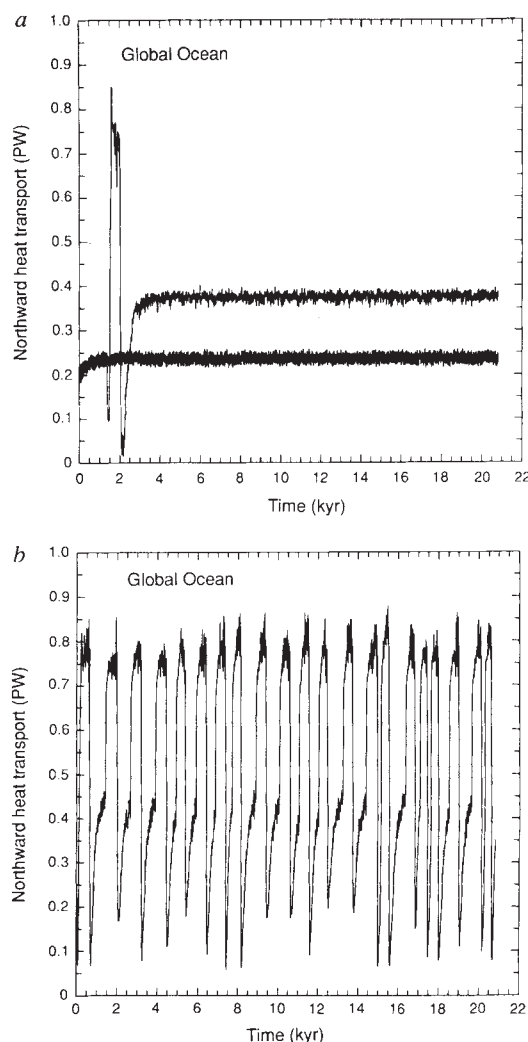


FIG. 4 The global ocean poleward heat transport at 28°N over the 20,800 yr of integration. *a*, The weak stochastic experiment (standard deviation, s.d. = 16 mm per month, lower curve) and the medium stochastic experiment (s.d. = 32 mm per month, upper curve). *b*, The strong stochastic experiment (s.d. = 48 mm per month).

trates these three different equilibria. In the first (Fig. 2*a*) the conveyor has a moderate 'present-day' strength of ~ 10 – 12 Sv (1 Sv is 10^6 m³ s⁻¹). The other equilibria correspond to a weakened ('colder'—Fig. 2*b*) and an enhanced ('warmer'—Fig. 2*c*) conveyor. We were not able to induce a stable equilibrium with North Pacific sinking. Indeed, in all three configurations of the Atlantic conveyor, the Pacific Ocean remained virtually unchanged.

The poleward heat transport in the global ocean and in the North Atlantic associated with the three equilibria are shown in Fig. 3*a* and *b*, respectively. In the 'present day' (normal) climate ~ 0.2 PW (1 PW is 10^{15} W) is transported northward at 28°N. This increases to 0.5 PW in the 'warmer' climate case associated with an intensified North Atlantic thermohaline circulation, and decreases to -0.03 PW in the 'colder' climate. The zonally-averaged North Atlantic oceanic surface heat flux, $Q(y)$, (where y is positive northwards) can be determined from these equilibrium heat transports, $T(y)$, as $dT/dy = Q(y)$. Hence the three equilibria correspond to normal, enhanced and decreased ocean-to-atmosphere heat fluxes in the North Atlantic ocean and hence, one would expect, normal, warmer and colder North Atlantic sea surface temperatures and climates, respectively.

The stability of the present-day climatic state (Fig. 2*a*) was then tested by adding a stochastic component with varying magnitude to the freshwater flux forcing field as in ref. 21. This procedure was adopted as a simulation of high-frequency atmosphere variability²². Four experiments were carried out with the standard deviation (s.d.) of the stochastic component being set to 16, 32, 48 or 64 mm per month corresponding to 20, 40, 60 and 80% of the globally-averaged annual mean precipitation^{23,24}. As the Eemian interglacial was, on average, slightly warmer than today³, we interpret the high-s.d. experiments as a representation of an enhanced hydrological cycle associated with the warmer climate. Recent coupled atmosphere–ocean simulations²⁵ of the climatic response to increasing atmospheric CO₂ have noted that the hydrological cycle intensifies as the climate warms, and hence our representation is quite reasonable.

When only a weak (s.d. = 16 mm per month) stochastic forcing was used, the 'present-day' overturning (Fig. 2*a*) remained largely unchanged (Fig. 4*a*). A Fourier spectrum of the curve shown in Fig. 4*a* revealed variability with peaks at both decadal and century timescales, linked to horizontal and overturning advective timescales, respectively²¹. When the stochastic forcing was increased to s.d. = 32 mm per month, the 'present-day' climate remained stable for $\sim 1,000$ yr, when the conveyor in the North Atlantic suddenly collapsed (to a state similar to Fig. 2*b*), then increased rapidly, collapsed again and then settled into the new 'warmer' climatic equilibrium (as in Fig. 2*c*). Superimposed on this climatic equilibrium was variability on both decadal and century timescales. In the s.d. = 48 mm per month integration (Fig. 4*b*) the system oscillated internally between states involving weak (minima in Fig. 4*b*) and strong (maxima in Fig. 4*b*) overturnings in the North Atlantic, passing through a period of normal (middle state in Fig. 4*b*) overturning along the way. The partitioning of time in each state is similar to that observed in the Eemian (Fig. 3 in ref. 1). At no time during any of the integrations was deep water formation found to occur in the Pacific Ocean. The transition between the states happened very rapidly (over several decades) and the system remained in a particular state for periods up to a thousand years or so. Once more, superimposed on the millennial-timescale variability was variability on both decadal and century timescales. The results of the final experiment (s.d. = 64 mm per month) were similar to those of the s.d. = 48 mm per month experiment, except that the frequency of catastrophic transitions increased and their magnitude decreased, consistent with the idealized single-basin study of ref. 21.

Although the model used here is idealized and so should be viewed as process-oriented rather than predictive, its results are significant because they suggest that climate variability during the last interglacial (which has been found in the GRIP ice-core data) may be due to fluctuations of the ocean's thermohaline circulation. Unlike previous theories of rapid climate fluctuations during glacial periods and during the transition between glacial and interglacial periods, our experiments did not involve continental ice/runoff–ocean interactions or major modifications of the Atlantic-to-Pacific hydrological cycle. One might speculate that such variability may well have also existed in glacial times although ice/runoff–ocean interactions must certainly be accounted for. For example, as shown in ref. 20, the occurrence of sudden ice-melt or freshwater run-off, or a modification of the hydrological cycle from the Atlantic to the Pacific Ocean, could indeed also induce transitions between equilibria consistent with these earlier theories and model results (for example, refs 26–29).

The stability of the 'present-day' model climate and associated weak decadal and century timescale variability under weak random forcing are also consistent with the observations in the present Holocene climate. Nevertheless, we are still left with the fundamental question of why the stability of our present Holocene would be different from that of the Eemian interglacial. We have suggested that the difference would be linked to an

enhanced hydrological cycle associated with the warmer³ mean climate of the Eemian, represented here as an increase in the magnitude of stochastic component of the freshwater flux forcing. We further suggest that this enhanced hydrological cycle would excite fluctuations of the North Atlantic conveyor between different modes of operation. If the variability found in the GRIP ice-core data is corroborated, then the Eemian interglacial may offer us a glimpse of the type of rapid climate variability which we might expect in a future climate warmed by means of anthropogenic greenhouse gas emissions. □

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The instability of a vaporization front in hot porous rock

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In many geothermal systems, water migrates into vapour-dominated porous rock either through natural recharge or through forced injection of water from a well^{1–5}. If the host rock is initially very hot, then a fraction of this injected water vaporizes^{6,7}; as the water injection rate increases, the fraction which vaporizes decreases⁷. For modelling purposes, it is generally assumed that liquid–vapour interfaces in hot porous rocks are planar and stable^{6,7}. But we show here, both theoretically and experimentally, that if a sufficient fraction of the liquid vaporizes, the interface can become unstable. The resulting ‘fingering’ instability can itself be stabilized: at short wavelengths by thermal diffusion, and at long wavelengths by the pressure increase caused by the excess vaporization at the tips of the fingers. These liquid fingers migrate through the porous rock much more rapidly than does a planar liquid front, and could therefore limit the time during which vapour may be extracted for geothermal power applications. We suggest that an optimal water injection rate for geothermal energy production may be that for which the interface is just stable, thereby maximizing the fraction of liquid which vaporizes, while suppressing the fingering instability.

High-temperature geothermal circulation systems involve complex liquid and vapour flow and phase change through permeable strata^{1,8}. Determination of the rate of vapour production as liquid invades a reservoir is crucial for optimizing geothermal power generation^{7,9,10}, for geochemical monitoring of the mixing of different fluid masses^{2,11}, for monitoring volcanic activity¹² and for understanding aspects of ore deposition^{13,14}. Vapour-dominated reservoirs, including the Geysers, California, are of particular interest because the extraction of vapour for power generation has led to a significant decline in the reservoir pressure and fluid levels^{15,16}. New vapour may be generated as water invades the reservoir and is heated by the hot rock. It is commonly assumed that in geothermal systems, such liquid–vapour interfaces migrating into the vapour-saturated region are stable and planar^{6,7}. This is motivated by the argument that a liquid finger advancing ahead of a liquid–vapour interface has

a larger surface area exposed to the hot rock, vaporizes more rapidly, and is thus resorbed into the front⁶. Indeed, in reservoirs of very low permeability, static liquid–vapour interfaces are stable to disturbances, even if the liquid overlies the vapour¹⁷. Moreover, the classical result of Saffman–Taylor¹⁸ shows that when a viscous fluid flows into a porous rock filled with a second fluid of lower dynamic viscosity, the interface remains stable and planar. In contrast, we show here that a migrating liquid–vapour interface may in fact become unstable.

The pressure gradient, G_1 , in liquid of dynamic viscosity μ_1 and density ρ_1 which flows through a liquid-saturated porous rock of permeability K with Darcy velocity u_1 is $G_1 = -\mu_1 u_1 / K$ (ref. 8). Suppose that a mass fraction f ($0 \leq f \leq 1$) of this liquid vaporizes and migrates ahead of the moving liquid–vapour interface. Then, by mass conservation, just ahead of the interface in the vapour-saturated region, this newly formed vapour has Darcy velocity $u_v = f u_1 \rho_1 / \rho_v$ and pressure gradient $G_v = -f \mu_v u_1 \rho_1 / K \rho_v$, where the vapour is of density ρ_v and dynamic viscosity μ_v . If the magnitude of the pressure gradient in the vapour exceeds that in the liquid, $|G_v| > |G_1|$, then, as with other migrating fluid–fluid interfaces^{17,19}, the interface can become

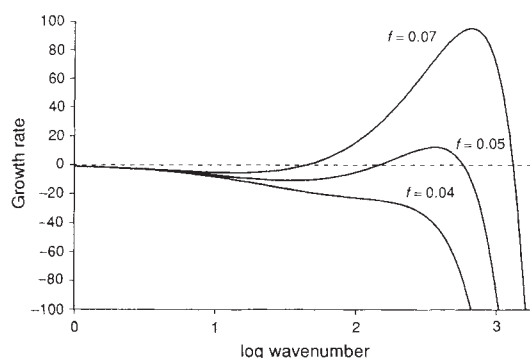


FIG. 1 Growth rate (σ) of a perturbation as a function of the wave-number (k). Curves are given for three values of the mass fraction vaporizing (f). In these calculations the thermal diffusivity $\kappa = 3 \times 10^{-6}$, $\kappa/\alpha = 5 \times 10^{-4}$ and $v_l/v_v = 0.042$. Note that Woods and Fitzgerald⁷ have shown that f lies in the range $0 < f < 0.8$ for injection of liquid water in a cylindrical geometry, with the value of f decreasing with injection rate, and that typically $v_l/v_v \approx 0.1$ – 0.2 in vapour-dominated geothermal reservoirs²⁰.

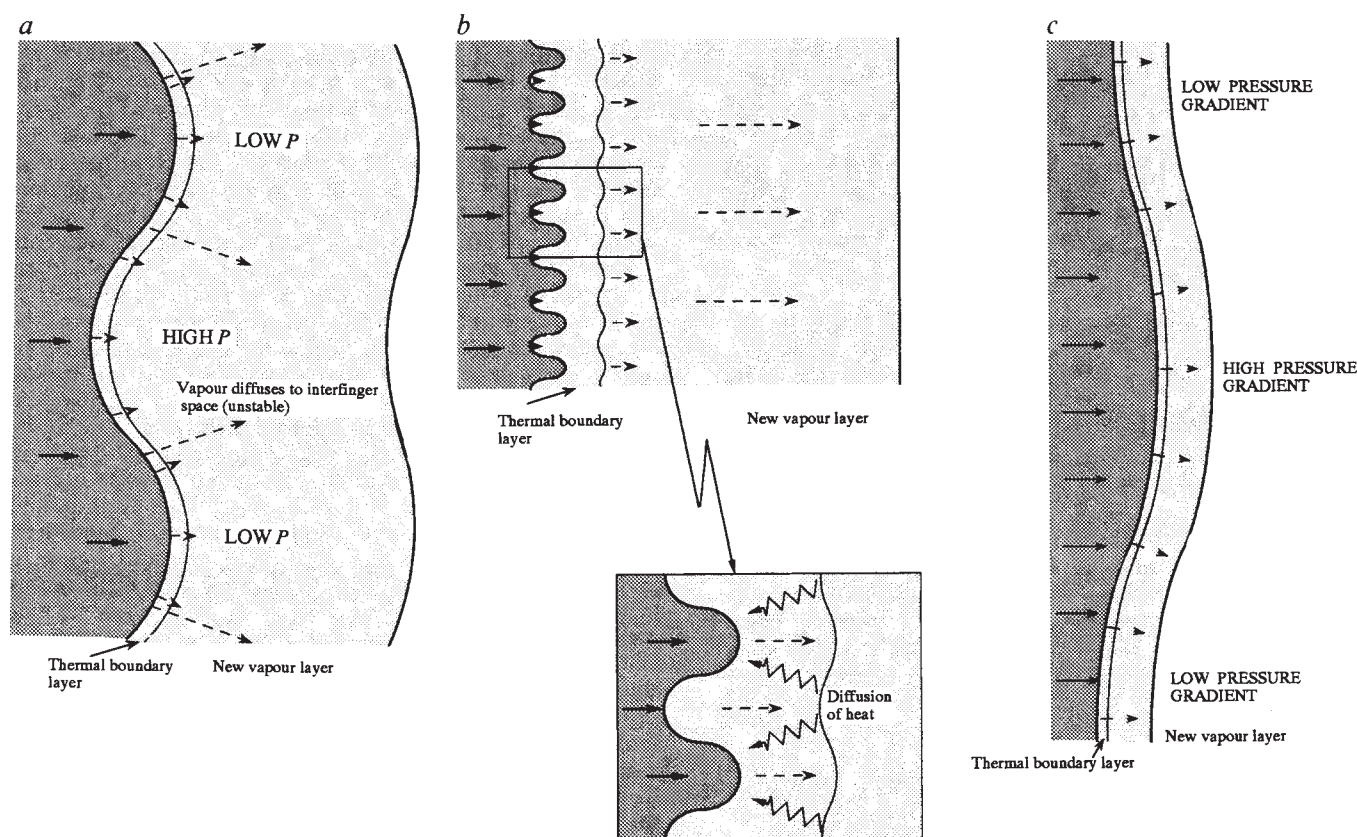


FIG. 2 Schematic diagrams showing: a, the development of an instability at a migrating vaporizing interface with a perturbation of intermediate wavelength; b, the suppression of the instability when the wavelength is too small owing to the effects of thermal diffusion, shown by the jagged arrows; c, suppression of the instability when the wave-

length is too large, owing to the differential vaporization between the tip and base of a finger. (P , pressure.) Solid arrows represent liquid flow, dashed arrows represent vapour flow and jagged arrows represent diffusion of heat.

unstable. Combining the above expressions we see that $|G_v| > |G_l|$ whenever

$$f > \left(\frac{\rho_v}{\mu_v} \right) \left(\frac{\mu_l}{\rho_l} \right) = \frac{v_l}{v_v} \quad (1)$$

Here v_l/v_v is the ratio of the kinematic viscosities of the liquid and vapour ($v = \mu/\rho$). For water, v_l/v_v lies in the range 0.1–0.2 depending upon the pressure and temperature²⁰. Therefore, if a sufficient fraction of the liquid vaporizes, the interface may become unstable. Woods and Fitzgerald⁷ have shown that the fraction of liquid which vaporizes, f , increases as the speed of the liquid supplied to the boiling front is decreased. For sufficiently slow injection rates, $f > 0.2$, and we deduce from equation (1) that the interface may become unstable. Note that in contrast, for oil, the ratio v_l/v_v has a value in the range 10^{-4} (refs 21, 22), and so a vaporizing oil–gas front is always stable.

We now extend the above physical argument with a detailed mathematical analysis of the stability of a steadily propagating, boiling interface. The vapour motion is described by the mass conservation equation and Darcy's law⁸

$$\phi \frac{\partial \rho_v}{\partial t} + \nabla \cdot (\mathbf{u}_v \rho_v) = 0; \quad \mathbf{u}_v = -\frac{K}{\mu_v} \nabla P \quad (2, 3)$$

together with the equation for the conservation of heat⁷ and an equation of state for the vapour²³

$$\frac{\partial (C_a T)}{\partial t} + \nabla \cdot (\mathbf{u}_v C_v T) = \kappa_a C_a \nabla^2 T; \quad P = \rho_v R_v T \quad (4, 5)$$

where C is a specific heat per unit volume, κ is the thermal diffusivity, subscript a denotes the average over the porous matrix and fluid, subscript v denotes vapour and R_v is the gas constant for vapour of temperature T and pressure P . Using these equations, Woods and Fitzgerald⁷ showed that the effective pressure diffusion coefficient $KP/\mu_v \phi$ is 10^2 – 10^4 times larger than the thermal diffusivity κ . Therefore, as new vapour migrates from the interface, it is heated to the far-field reservoir temperature in a narrow thermal boundary layer just ahead of the interface; in contrast, the vapour pressure adjusts to the far field across a much wider pressure boundary layer. The flow in the liquid is incompressible, and is governed by Darcy's law⁸. Owing to the thermal inertia of the hot porous medium³, we assume the liquid attains the interface temperature before reaching the interface^{6,7}. Mass and heat conservation across the boiling interface, located at $z = \eta(x, y, t)$ are given by

$$\phi (\rho_l - \rho_v) \frac{dz}{dt} = \rho_l u_l - \rho_v u_v \quad (6)$$

$$\phi (h_l - h_v) \frac{dz}{dt} = \left[\kappa_a C_a \frac{\partial T}{\partial z} \right] + u_l h_l - u_v h_v \quad (7)$$

where $[x]$ denotes the change in the property x across the interface, h denotes specific enthalpy, and subscript l represents liquid. At the interface, the vapour is in thermodynamic equilibrium with the liquid, as specified by the Clausius–Clapeyron relation²⁰.

Following Woods and Fitzgerald⁷, we constructed simple analytical solutions for the steady propagation of a planar boiling

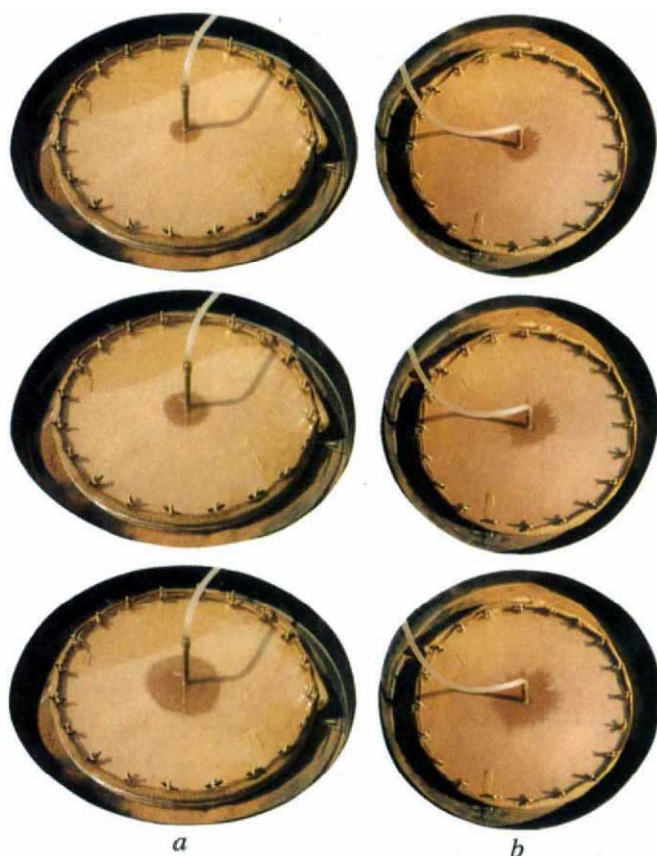


FIG. 3 *a* (left-hand column), *b* (right-hand column), Photographs of two experiments in which a boiling front of ether propagated radially through a porous sand layer of depth 2 cm, radius 25 cm and an estimated permeability of 10^{-12} m². *a*, Sequence of three pictures of a low-temperature sand layer in which the interface remains circular, and no instability forms. *b*, High-temperature sand layer in which the interface develops a fingering instability. Owing to the short duration of the experiments, the perspex plates acted as insulators. Photographs were taken at intervals of 10–15 s.

front, with speed $dz/dt = u$, by combining equations (2–7) and linearizing the resulting equations⁶. Using standard perturbation theory^{24,25} we now examine the linear stability of these steady solutions to small perturbations of the form

$$\eta \rightarrow \eta + \varepsilon \exp(\sigma t + ikx)$$

$$P(\chi) \rightarrow P(\chi) + \varepsilon a(\chi) \exp(\sigma t + ikx)$$

$$T(\chi) \rightarrow T(\chi) + \varepsilon b(\chi) \exp(\sigma t + ikx)$$

where $\chi = z - ut$, $\varepsilon \ll 1$, k is the wavenumber, $2\pi/k$ is the wavelength and σ is the growth rate of the perturbations. In Fig. 1, the growth rate of perturbations, σ , is plotted as a function of the wavenumber, k , for three values of the mass fraction vaporizing, f .

Figure 1 shows that if f is too small, the interface remains stable ($\sigma < 0$), in accord with our earlier physical discussion (equation (1)); indeed, for the physical parameters of Fig. 1, equation (1) implies absolute stability if $f < 0.042$. However, for sufficiently large f there is a range of unstable wavelengths, that is, for which $\sigma > 0$. For perturbations whose wavelength is in the range leading to instability, $\sigma > 0$, the surface area of the interface is sufficiently large that additional vapour, produced by the advancing fingers, can migrate freely to the side of the fingers (Fig. 2*a*). But for the same value of f , perturbations of

wavelength smaller than the width of the narrow thermal boundary layer ahead of the interface (large k) are stabilized ($\sigma < 0$) by diffusion of heat from the hot rock (Figs 1 and 2*b*). (Note that this stabilization mechanism has some similarity to the short-wavelength stabilization of viscous fingers between miscible fluids by the transverse diffusion of species²⁶.) For perturbations of wavelength longer than the width of the high-pressure boundary layer ahead of the interface (small k), the newly formed vapour accumulates just ahead of the interface rather than diffusing into the inter-finger spaces (Fig. 2*c*). Because the pressure is lower at the tips of the fingers than at their base, more vapour is produced at the tip⁷. As a result, the pressure at the tips of the fingers increases more rapidly than at their base and this stabilizes the interface (Figs 1 and 2*b*). (Note that this stabilization mechanism is analogous to the long-wavelength morphological stability of solid growing from a binary melt²⁵.)

In order to demonstrate that instability can indeed occur, we conducted a series of experiments in which ether was injected into a cylindrically symmetric porous layer of hot sand, bounded by two clear perspex plates (Fig. 3). For ether, $v_l/v_v \approx 0.07$ (ref. 21). As the ether spread radially from the central source, a boiling front developed and ether vapour migrated ahead of this interface, issuing from the edge of the cylinder a few seconds after the start of the experiment. When the sand was relatively cool, of the order of 50 °C, the mass fraction of ether which vaporized was small, < 0.07 , and the advancing liquid ether front remained stable and circular (Fig. 3*a*). In contrast, for a higher initial sand temperature, of the order of 150 °C, a much larger fraction of the ether vaporized, and the liquid front developed fingers (Fig. 3*b*). This instability evolved rapidly to a highly nonlinear regime.

This instability has a number of important implications. If vapour is to be extracted from a geothermal reservoir, liquid must be injected to regenerate the vapour, but the time for this liquid to reach the extraction site must be maximized. At a planar liquid interface, the pressure is typically several bars greater than the far-field vapour pressure⁷. In contrast, the pressure at the tip of a developed liquid finger is much lower because the newly formed vapour can diffuse to the side of the finger, rather than accumulating ahead of it. Thus liquid fingers can travel to the extraction site much faster than a planar liquid front. Fingering should therefore be suppressed; this requires a low vaporizing fraction f (equation (1)) and a high injection rate⁷. The optimal water injection rate may be that for which the interface is just stable, as this also maximizes the fraction of injected liquid converted to vapour. Finally, we note that if a boiling front develops fingers, then the deposition patterns of minerals precipitated at the front (for example, quartz and precious metals^{13,14}) may be highly irregular and vein-like. □

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Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart

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INHERITED rickettsial symbionts of the genus *Wolbachia* occur commonly in arthropods and have been implicated in the expression of parthenogenesis^{1,2}, feminization^{2,3} and cytoplasmic incompatibility phenomena in their respective hosts^{4–7}. Here we use purified *Wolbachia* from the Asian tiger mosquito, *Aedes albopictus*, to replace the natural infection of *Drosophila simulans* by means of embryonic microinjection techniques. The transferred *Wolbachia* infection behaves like a natural *Drosophila* infection with regard to its inheritance, cytoskeleton interactions and ability to induce incompatibility when crossed with uninfected flies. The transinfected flies are bidirectionally incompatible with all other naturally infected strains of *Drosophila simulans*, however, and as such represent a unique crossing type. The successful transfer of

this symbiont between distantly related hosts suggests that it may be possible to introduce this agent experimentally into arthropod species of medical and agricultural importance in order to manipulate natural populations genetically.

The expression of cytoplasmic incompatibility (CI) is associated with disruptions to early fertilization events in the insect host^{8–11}, which for most species is lethal. Incompatibility is most commonly seen when infected and uninfected insects are crossed^{12–14} and more rarely between different insect strains that both harbour *Wolbachia* infections. In the latter case the incompatibility can be observed in one or both directions of a cross, generating complex mixtures of CI crossing types within and between species^{8,11,15–18}. The basis for incompatibility between two infected populations has long been considered to relate to infection with different strains of rickettsial symbiont⁴, although recent phylogenetic studies based on *Wolbachia* 16S ribosomal RNA gene sequences in *Drosophila* have found no correlation between bacterial phylogeny and crossing type⁵. Furthermore, recent observations in *Nasonia* suggest that differing densities of the same *Wolbachia* strain can influence unidirectional crossing types¹⁶.

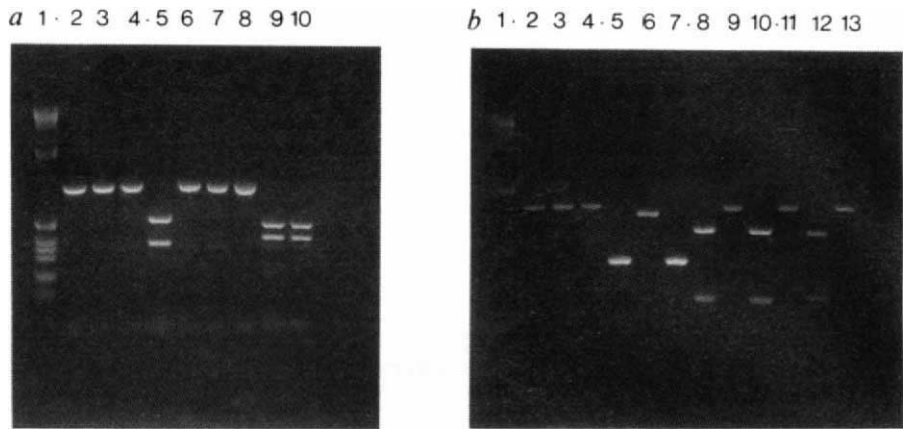
To clarify the role of bacterial strains in the expression of CI crossing types, we used embryonic microinjection techniques to replace the natural bacterial symbiont of a well characterized strain of *D. simulans* from Riverside, California (DSR)^{11,12,17} with an *Ae. albopictus* counterpart. A derivative of DSR that had been previously cured of its *Wolbachia* symbiont by tetracycline treatment (DSRT) was used as the recipient in microinjection

TABLE 1 Egg mortality in single pair crosses between *D. simulans* transinfected with *Wolbachia* from *Ae. albopictus*, a tetracycline-cured strain, and two naturally infected strains from Riverside and Hawaii

Cross (♀ × ♂)		Percentage egg mortality $\bar{x}$	s.e.m.	Total eggs counted	Number of crosses n	Comparison	Probability t-test P
Experiment 1							
DSR(Aa) × DSR(Aa)	a	18.3	8.3	687	16		
DSR(Aa) × DSRT	b	5.4	1.5	285	6	b, a	NS
DSRT × DSR(Aa)	c	67.5	9.1	659	18	c, d	<0.001
DSRT × DSRT	d	3.9	1.4	776	16		
Experiment 2							
DSR(Aa) × DSR(Aa)	e	14.8	4.0	1,792	25		
DSR(Aa) × DSR	f	79.4	3.4	1,664	24	f, e	<0.001
DSR × DSR(Aa)	g	90.1	1.7	1,358	16	g, h	<0.001
DSR × DSR	h	2.8	0.7	1,434	20		
DSR(Aa) × DSH	i	80.2	4.4	1,729	25	i, e	<0.001
DSH × DSR(Aa)	j	93.2	1.3	3,899	35	j, k	<0.001
DSH × DSH	k	12.4	5.7	962	16		
DSR × DSH	l	70.3	7.4	784	15	l, h	<0.001
DSH × DSR	m	93.1	1.2	2,794	25	m, k	<0.001

Abbreviations: DSR: *D. simulans* Riverside, CA; DSRT: *D. simulans* Riverside, tetracycline-treated¹¹; DSR(Aa): DSRT strain transinfected with *Wolbachia* from *Ae. albopictus*, Houston, Texas; DSH: *D. simulans* Hawaii; $\bar{x}$: mean; s.e.m.: standard error of the mean; NS: not significant, $P > 0.05$. Crossing: Crosses were established between appropriate individuals and left for two days before the female was put in a flask with a removable molasses plate (30% molasses, 2% agar) supplemented with liquid living yeast. Plates were exchanged after 24 h, the eggs counted and then incubated for 48 h, and the hatching larvae counted. Females that had laid less than 10 eggs were not included in the analysis. Females from crosses in which no eggs hatched were checked for insemination by dissection and examination of spermathecae. Unmated females were excluded from analysis. *Wolbachia* infection was confirmed in transinfected males used in experiment (2) crosses by PCR analysis. Crosses involving uninfected males were excluded¹⁹. Injections: *Wolbachia pipiensis* was crudely purified from 24-h-old *Ae. albopictus* eggs. The mosquito eggs were surface-disinfected with 50% Clorox for 2 min, washed, and homogenized in 30% sucrose in PBS. The suspension was freed from cellular debris by microcentrifugation: 3 times for 1 min and once for 5 min at 3,000g, then *Wolbachia*-pelleted for 5 min at 7,000g; the pellet was resuspended in 10 μ l Ringer's solution. Recipient DSRT eggs were less than 45 min old, dechorionated with 50% Clorox and microinjected posteriorly with glass needles having tip diameters of 6–12 μ m. After hatching, larvae were fed on rearing diet until eclosion. Virgin females were isolated and mated to DSRT males in order to establish isofemale lines.

FIG. 1 Restriction fragment-length polymorphisms (RFLPs) of PCR-amplified 16S and 12S ribosomal subunit DNA fragments were used to determine the origin of both the *Wolbachia* (a) and mitochondria (b) in the transinfected DSR(Aa) line. a, Lanes: 2, 5 and 8, DSR; 3, 6 and 9, *Ae. albopictus*; 4, 7 and 10, DSR(Aa); 2, 3 and 4, uncut *Wolbachia* 16S fragments were amplified with specific 76–99 forward and 1,012–994 reverse primers as described⁷; 5, 6 and 7, digested with *Xba*I, diagnostic for *Wolbachia* from DSR; 8, 9 and 10, digested with *Ava*I, diagnostic for *Wolbachia* from *Ae. albopictus*. Lane 1, 1-kb ladder (Gibco/BRL). The RFLP patterns generated with these enzymes show that DSR(Aa) contains *Wolbachia* originating from *Ae. albopictus*. b, Lanes: 1, 1-kb ladder (Gibco/BRL); 2, 5, 8 and 11, DSR; 3, 6, 9 and 12, *Ae. albopictus*; 4, 7, 10 and 13, DSR(Aa); 2, 3 and 4: uncut fragments; 5, 6 and 7, digested with *Ssp*I, diagnostic for mitochondria from DSR; 8, 9 and 10, digested with *Xba*I, diagnostic for mitochondria from *Ae. albopictus*; 11, 12 and 13, digested with *Hinf*I, diagnostic for mitochondria from DSR. The mitochondrial 12S rDNA fragments were amplified with



12SAI and 12SBI primers^{7,24}. PCR amplifications were performed as described⁷, then ethanol-precipitated and digested with the appropriate enzyme. 16S fragments were resolved on a 2% Seakem LE gel and 12S fragments on a 3% 3:1 Nusieve gel; both were stained with ethidium bromide and visualized under ultraviolet light.

experiments¹¹. This strain was confirmed as being free of symbionts before injection using a diagnostic polymerase chain reaction (PCR) assay for *Wolbachia* infection⁷. Purified *Wolbachia* fractions were prepared from the mosquito *Ae. albopictus* and microinjected into *D. simulans* embryos before pole cell formation (Table 1 legend).

Injected lines were monitored at each generation for the presence of *Wolbachia* using the specific 16S-rRNA-based PCR assay⁷. After six generations of monitoring, one isofemale line tested positive for the presence of *Wolbachia*. Diagnostic restriction endonuclease sites within the amplified 16S rRNA PCR product of this line were used to confirm that this infection was due to the successful transfer of the *Ae. albopictus* symbiont and not to a resurgence of the natural *Drosophila* infection in the tetracycline-treated flies (Fig. 1a). To determine whether this transinfected line was capable of inducing CI, test crosses were established between the transinfected line and the parental cured

strain. Crosses between transinfected males and uninfected females resulted in 68% egg mortality (Table 1), which is significantly higher than controls and indicative of CI expression. However, the egg mortality rates measured in this cross are lower than normally seen in naturally infected DSR-incompatible crosses¹⁹, and probably reflect lower densities of *Wolbachia* in the transinfected line at the time this cross was made. Similar values have been reported during the establishment of intra-specific transinfected DSR lines¹⁹.

To determine what effect the transferred symbiont had on *Drosophila* crossing types, test crosses were established between the transinfected DSR(Aa) line, the naturally infected DSR line and a strain of *Drosophila* originating from Hawaii (DSH), known to be bidirectionally incompatible with DSR¹¹. The transinfected DSR(Aa) line proved to be bidirectionally incompatible with the naturally infected DSR from which it was derived, and also bidirectionally incompatible with the DSH line (Table 1).

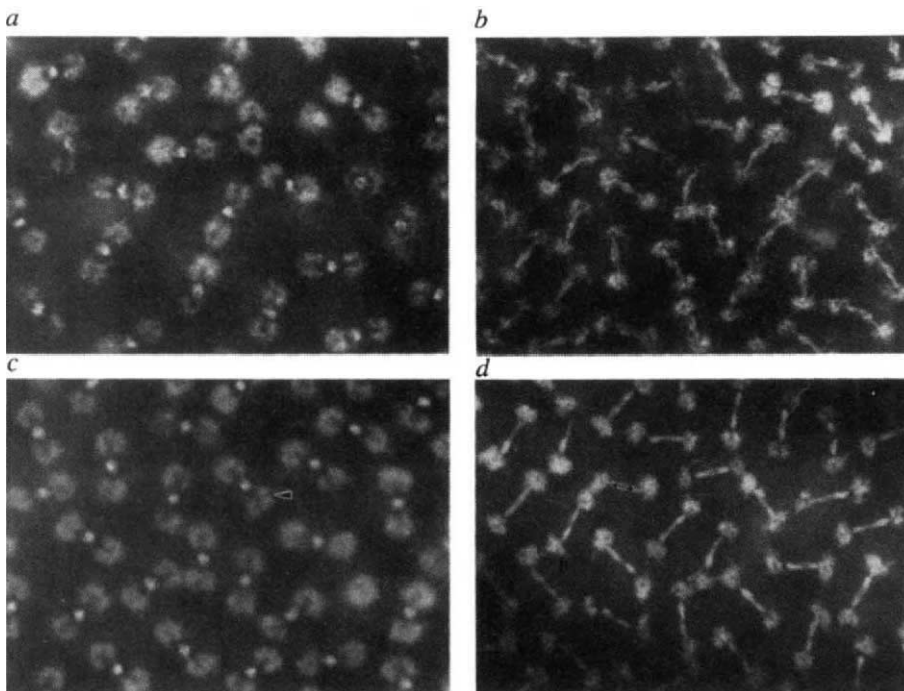


FIG. 2 DAPI staining of *Drosophila* embryos undergoing early synchronized mitotic divisions. a and b, DSR embryos at metaphase (a) and anaphase (b) showing localization of bacterial symbionts to spindle poles. c and d, DSR(Aa) transinfected embryos showing similar localization of the *Ae. albopictus* symbiont in its new host. Arrowhead in c indicates *Wolbachia* and arrows in d indicate host chromosomes and spindle axis. Methods have been described¹¹.

As such, the transinfected DSR(Aa) line displays a unique *Drosophila* crossing type.

The mitochondrial composition of the transinfected flies was determined using diagnostic restriction-fragment length polymorphisms (RFLPs) of a PCR-amplified region of the mitochondrial 12S rRNA gene (Fig. 1b). Initially DSR(Aa) appeared heteroplasmic for both *Ae. albopictus* and *D. simulans* mitochondria, but by the tenth generation the strain had reverted to a homoplasmic state consisting of only *D. simulans* mitochondria. To verify that the strain was completely homoplasmic, a sample of flies from the transinfected strain used in crosses reported in Table 1 (experiment 2) were used as a source of template DNA for five independent PCR amplifications of the 12S gene fragment. The resulting DNA fragments were pooled and cloned using a t-tailed phagemid²⁰. Sixty-one clones containing the 12S rRNA insert were screened with the diagnostic *Hinf*I enzyme and all had a restriction profile characteristic of *Drosophila* mitochondrial DNA (data not shown). Therefore, the DSR(Aa) line represents a transfer of *Ae. albopictus Wolbachia* into a DSR mitochondrial background. These results show that host mitochondrial genotype does not determine the expression of bidirectional incompatibility, in accord with earlier cytoplasmic transfer experiments in *Drosophila*¹⁸.

Besides having profound effects on host karyogamy, natural *Wolbachia* infections of *Drosophila* also display an intriguing association with the host cytoskeleton¹¹. This is particularly striking in the early mitotic divisions of developing *Drosophila* embryos, when bacterial symbionts are readily seen interacting with the host spindle apparatus¹¹ (Fig. 2a, b). Bacterial localization within transinfected embryos was compared with their naturally infected DSR counterparts by DAPI (4,6-diamidino-2-phenylindole) staining¹¹ and subsequent examination by epifluorescent microscopy. The transinfected *Ae. albopictus* symbiont behaved in a similar manner within the young *Drosophila* embryo, being concentrated in the egg cortex region and localized towards the host centrosome during mitosis (Fig. 2c, d). Therefore the transferred *Wolbachia* infection effectively behaved as a natural *Drosophila* infection in terms of its inheritance, ability to induce CI, and also in its cytoskeleton associations.

These studies clearly show that different bacterial strains are associated with the expression of bidirectional CI crossing types. Furthermore, they demonstrate that while these bacterial symbionts are capable of elaborate interactions with their insect host, as evidenced from their cytoskeleton associations and influence on host karyogamy, these interactions do not reflect the evolution of an obligate dependence of bacterial strain for a specific insect host. Our results complement previous phylogenetic studies indicating that the *Wolbachia* symbionts of different insect hosts are very closely related and have probably moved horizontally between insect lineages in the past^{2,5,7}. The demonstration that these agents can be experimentally transferred over large phylogenetic distances and that they can form a stable inherited infection that can induce a new CI phenotype in a foreign host suggests that it may be possible to transfer *Wolbachia* into insect disease vectors and insects of economic importance in order to manipulate natural populations genetically^{21, 23}. □

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Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning

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NEURAL-CELL adhesion molecules (N-CAMs) are members of the immunoglobulin superfamily mediating homo- and heterophilic cell-cell interactions. N-CAM exists in various isoforms which are generated by alternative splicing^{1–3}. During embryonic development, N-CAMs are expressed in derivatives of all three germ layers, whereas in the adult animal they are predominantly present in neural tissue. Processes like neurulation⁴, axonal outgrowth⁵, histogenesis of the retina^{6,7} and development of the olfactory system^{8–10} are correlated with the regulated expression of N-CAMs^{11–14}. We show here that N-CAM-deficient mice generated by gene targeting appear healthy and fertile, but adult mutants show a 10% reduction in overall brain weight and a 36% decline in size of the olfactory bulb. N-CAM deficiency coincides with almost total loss of protein-bound α -(2,8)-linked polysialic acid, a carbohydrate structure thought to be correlated with neural development and plasticity^{15,16}. The animals showed deficits in spatial learning when tested in the Morris water maze¹⁷, whereas activity and motor abilities appeared normal.

Gene targeting and generation of homozygous mutant mice were performed using standard protocols¹⁸ (Fig. 1A), and confirmed using Southern blotting (Fig. 1B) and the allele-specific polymerase chain reaction (PCR; Fig. 1C). Nuclease S1 protection assays (Fig. 1D) and northern (not shown) and western blotting (Fig. 1E) confirmed the targeted locus as a null allele. Immunocytochemical analysis of brain sections for N-CAM using monoclonal antibodies and polyclonal sera showed most intense staining in the glomeruli and granular cell layer of the olfactory bulb in wild-type and heterozygous animals (Fig. 2b). Overall staining agreed well with reports on N-CAM expression in the adult brain^{10,16,19,20}. Homozygous mutant mice showed a total loss of N-CAM immunoreactivity (Fig. 2b), as expected.

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Heterozygous animals of two lines were mated and produced 78 offspring. Of these, 38 animals (49%) were heterozygous, 22 (28%) were wild type and 18 (23%) were homozygous for the mutated allele, indicating an almost perfect mendelian distribution. Homozygous mutant animals are fertile and appear healthy

for up to four months of age, even though they weigh about 10% less than wild-type and heterozygous littermates (data not shown). Figure 2a shows isolated brains of a mutant and a heterozygous animal, indicating the two most obvious anatomical differences: first, the olfactory bulb is smaller in mutants

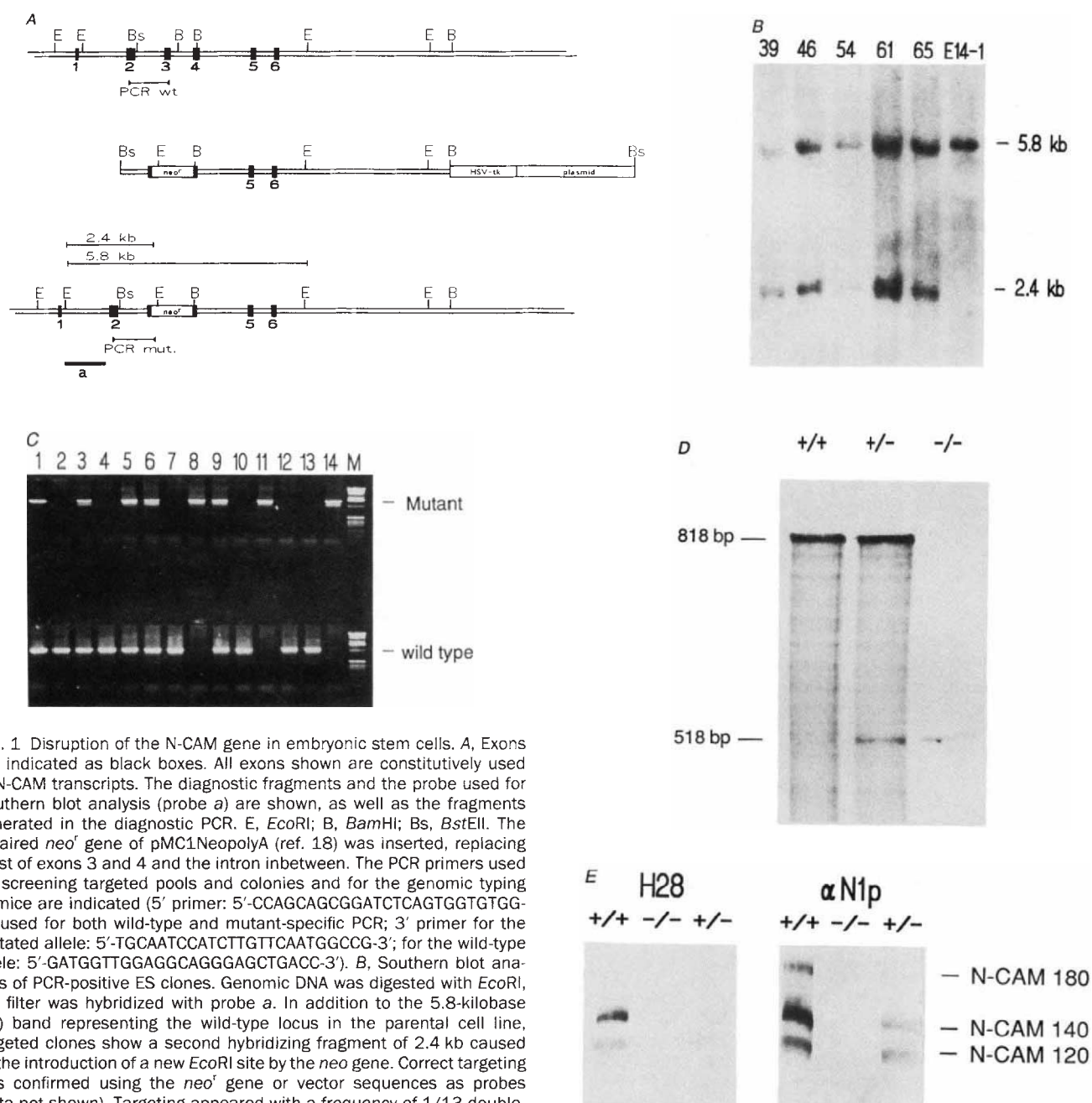


FIG. 1. Disruption of the N-CAM gene in embryonic stem cells. A, Exons are indicated as black boxes. All exons shown are constitutively used in N-CAM transcripts. The diagnostic fragments and the probe used for Southern blot analysis (probe a) are shown, as well as the fragments generated in the diagnostic PCR. E, *EcoRI*; B, *BamHI*; Bs, *BstEII*. The repaired *neo*^r gene of pMC1NeopolyA (ref. 18) was inserted, replacing most of exons 3 and 4 and the intron inbetween. The PCR primers used for screening targeted pools and colonies and for the genomic typing of mice are indicated (5' primer: 5'-CCAGCAGCGGATCTCAGTGGTGTGG-3', used for both wild-type and mutant-specific PCR; 3' primer for the mutated allele: 5'-TGCAATCCATCTTGTTCATGGCCG-3'; for the wild-type allele: 5'-GATGGTTGGAGGCAGGGAGCTGACC-3'). B, Southern blot analysis of PCR-positive ES clones. Genomic DNA was digested with *EcoRI*, the filter was hybridized with probe a. In addition to the 5.8-kilobase (kb) band representing the wild-type locus in the parental cell line, targeted clones show a second hybridizing fragment of 2.4 kb caused by the introduction of a new *EcoRI* site by the *neo* gene. Correct targeting was confirmed using the *neo*^r gene or vector sequences as probes (data not shown). Targeting appeared with a frequency of 1/13 double-resistant colonies. C, Allele-specific PCR analysis performed with tail DNA from offspring of a heterozygous cross. Animals 1, 3, 5, 6 and 9 are heterozygous, 2, 4, 7, 10, 12 and 13 are wild type and 8, 11 and 14 are homozygous mutant. The generated fragments are indicated in A. M, size marker. D, Nuclease S1 protection assay² for the analysis of transcripts derived from the mutant and wild-type locus. Total brain RNA was hybridized to a single-stranded DNA probe spanning the mutated sequences. The 818-bp fragment represents the unmutated transcript which is present in wild-type and heterozygous animals. The 518-bp signal appears in heterozygous and homozygous mutant animals, showing the 5' part of the mRNA to the nucleotide where the *neo*^r gene is introduced in exon 3. No additional signals that would indicate alternative or cryptic splicing or read-through transcripts can be seen. E, Western analysis of mutant animals. Protein extracted from brain tissue underwent SDS-PAGE and was stained with the monoclonal anti-N-CAM

antibody H28 (gift from C. Goriadis) or polyclonal rabbit serum (α N1p). In neither case was staining seen with protein of (-/-) animals. METHODS. E14-1 cells (subclone of line E14; gift from M. Hooper) were grown on mitomycin C-treated, *neo*-resistant primary embryonic fibroblasts. Transfection was performed with 25 μ g ml⁻¹ linearized vector as described¹⁸. Several PCR-positive clones were expanded, further analysed by Southern blotting and injected into C57Bl/6 embryos. Allele-specific PCR was performed in two separate reactions for each animal. Nuclease S1 protection assay was performed as described² with a single-stranded probe hybridizing to exons 0-5 of all N-CAM transcripts. For western analysis of N-CAM, protein was isolated from total brain tissue and 20 μ g were subjected to SDS-PAGE. As secondary antibodies, alkaline phosphatase-conjugated anti-rat (H28) or anti-mouse (α N1p) antibodies were used.

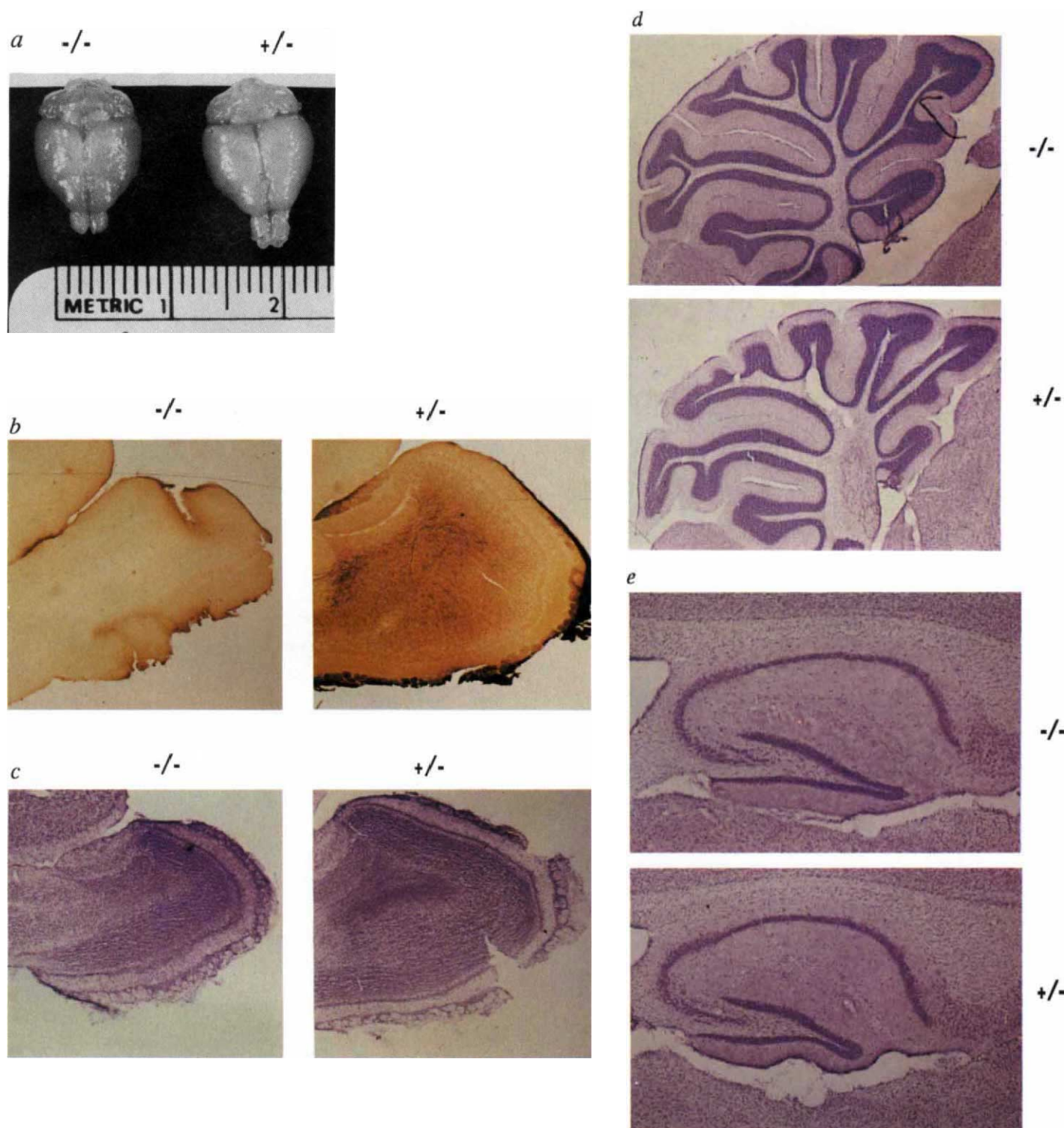


FIG. 2 *a*, Brains isolated from mutant and heterozygous adult mice. A reduction in the size of the olfactory bulb is visible. Brains were dissected from the skull after perfusion. *b*, Sections of the olfactory bulb of a heterozygous and a homozygous mutant adult animal. Staining was performed with the monoclonal anti-N-CAM antibody H28 which recognizes all N-CAM forms. Most intense staining in heterozygous mice is seen in the glomeruli and the granule cell layer. There is a total loss of staining in the mutant olfactory bulb, as expected. *c*, Cresyl violet staining for Nissl substance of the olfactory bulb. The decline in size of the olfactory bulb in mutants depends mainly on a reduction of the granule cell layer. The size of the glomeruli, the diameter of the external and internal plexiform layer and the mitral cell layer remains the same in +/- and -/- animals. *d*, Cresyl violet staining of the cerebellum. No difference in structure or size of the cerebellum was detected between

heterozygous and homozygous mice. *e*, Cresyl violet staining of the hippocampus. As in the cerebellum, no difference was detected between the two groups concerning cytoarchitecture or size of the hippocampus. The hippocampus was chosen for area measurements for comparison with the olfactory bulb.

METHODS. The animals were given an overdose of sodium pentobarbital and perfused through the heart with a 4% solution of paraformaldehyde in phosphate buffer. The brains were postfixed in 30% sucrose and frozen sagittal sections were taken. Three cresyl violet-stained sections of each animal were subjected to area measurements using an image analysis programme (Image 1.5; NIH) on a Macintosh Quadra 950 interfaced with a Leitz Orthoplan microscope. Antibody staining used biotinylated secondary antibodies and the Vectastain ABC kit (Vector) before reaction with diaminobenzidine and H_2O_2 .

than in $+/+$ and $+/-$ animals; and second, the brain weight is about 10% less (after correction for body weight).

Closer examination of the brain structure using sections stained by different methods and antibodies against marker genes²¹ revealed a normal cytoarchitecture (Fig. 2b–e). Areal measurements showed a 36% smaller olfactory bulb in homozygous mutants, although the size of the hippocampus was unaltered. The size reduction of the olfactory bulb seems to be caused mainly by the smaller size of the granular cell layer, one of the two areas showing the strongest immunostaining for N-CAM in wild-type and heterozygous animals (Fig. 2b). All other structures of the olfactory bulb appear normal in size and cytoarchitecture.

The olfactory bulb contains two differently glycosylated forms

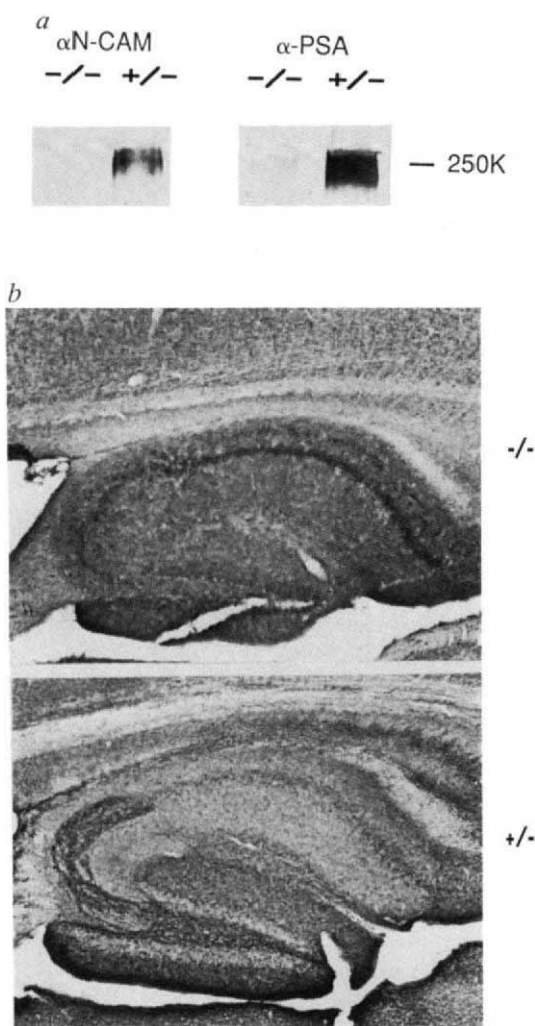


FIG. 3 a, Western blot analysis of total brain protein from neonatal mice for the presence of PSA. Staining was performed with anti-N-CAM antibody H28 and monoclonal anti-PSA antibody 735 (gift from R. Gerhardt-Schahn). With the disappearance of N-CAM staining in homozygous mutant mice, most of the PSA staining in these mice is removed. The blot was analysed densitometrically using a Joyce-Loebl Chromoscan III. b, Staining of hippocampus sections of adult $+/-$ and $-/-$ mice for PSA. There is an intense immunoreactivity of the mossy fibres in the heterozygous animals and a total lack of such staining in homozygotes, whereas the pyramidal cells in the CA1 region remain positive for PSA. The overall, slightly darker appearance of the mutant section is due to an overstaining.

METHODS. Western blot analysis was performed as described for Fig. 1; immunohistochemistry is described in Fig. 2 legend.

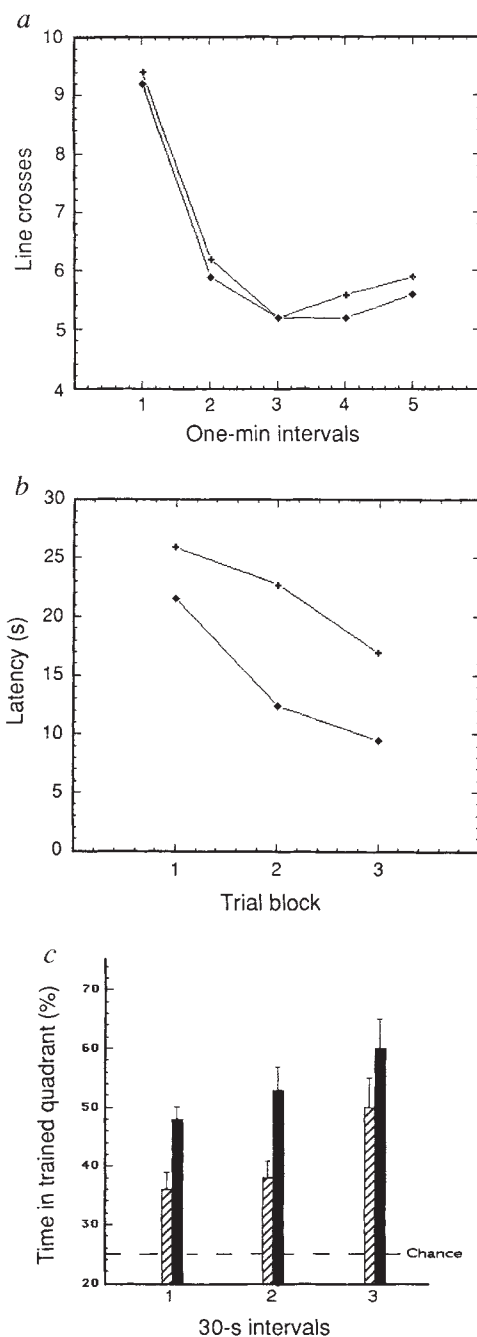


FIG. 4 a, Activity in the open field test. In this test for activity and motor abilities, both groups of animals showed a steady decline in activity over the 5-min test period and did not differ from each other ($F(1,24) = 0.13$, $P > 0.73$), indicating that the differences in the water maze task are not attributable to motor deficits. $\blacklozenge$, Control; $+$, mutant. b, Mean escape latencies in the Morris water maze. Mutant (+) and control ($\blacklozenge$) animals were trained to find a hidden platform in a circular pool by extra-maze cues. Each mouse completed three blocks of four acquisition trials. As shown in ref. 28, rats reach lowest search latencies after 8–12 trials. A group $\times$ trial blocks analysis of variance indicated that the decline in mean latency over trial blocks differed significantly between both groups ($F(1,24) = 6.6$, $P < 0.02$). Planned comparisons conducted at each trial block revealed further that latencies for mutants and controls were not significantly different for trial block I ($t(24) = 1.1$, $P > 0.29$), but were significantly different for trial blocks II ($t(24) = 2.3$; $P < 0.03$) and III ($t(24) = 2.5$, $P < 0.02$). c, Mean search time in the target quadrant in the probe trial following acquisition. Mutant mice (hatched) spent significantly less time in the target quadrant than controls ($\blacksquare$) ($F(1,23) = 7.5$, $P < 0.02$) during the three consecutive 30-s intervals of the probe trial. The reasons for the prolonged search time of both groups during the last 30-s interval are unknown.

of N-CAM²⁰, a highly α -(2,8)-polysialylated (PSA) form mainly expressed in the granular cell layer, and a less sialylated form heavily expressed in the glomeruli. The fact that only the structure expressing large amounts of PSA, the granular cell layer, is reduced in N-CAM-deficient and thus PSA-poor mice, might reflect the lack of PSA rather than of N-CAM. In cell culture experiments, the removal of PSA from the plasma membrane increases the area of cell contact in brain cell aggregates and affects cell adhesion properties²².

Unlike most other brain regions, the olfactory system maintains a high degree of plasticity even in adults²³. It is also the major region of the brain in which N-CAM proteins appear in their highly polysialylated form¹⁰ in the adult. N-CAM isoforms were shown to delineate the olfactory nerve in a spatially restricted pattern^{8,9}, suggesting an important role in the regenerative capability of this system. The fact that after elimination of N-CAM the most obvious change in the nervous system occurs in the olfactory bulb strengthens the view that N-CAM is involved in the formation and regeneration²⁴ of neural connections.

To analyse the relative amount of PSA on N-CAM compared with other proteins, we stained total brain protein of neonatal mutant and control mice for the presence of these sugar chains (Fig. 3a). Western blot analysis showed that N-CAM deficiency produces an 85% loss of total PSA content which is thus not compensated for by sialylation of other proteins.

The reduced size of the olfactory bulb in mutants raised the question of whether they can smell. Thus, mutant and control mice underwent a test in which they had to choose between 'attractive' bedding from their own cage and repellent bedding sprayed with lemon juice²⁵. Over a 3-min period, both groups of animals spent significantly less time over the lemon-flavoured half of the test field (control, 63 s, $n=15$; mutant, 62 s, $n=10$), demonstrating that mutants can distinguish between different odours. The overall activity and motor abilities of mutant animals were assessed using an open field test. Mutants and controls showed a similar steady decline in activity over the test period (Fig. 4a).

Recent observations in chicken²⁶ and *Aplysia*²⁷ suggest that N-CAM and related molecules might be involved in processes like learning and memory. To test N-CAM-mutant mice for their spatial learning ability, we performed a modified version of the Morris water maze (ref. 17, and P.K. and C. Randall, manuscript submitted). Mutant and control animals did not differ significantly in their escape latencies in trial-block I, but differed significantly in sessions II and III (Fig. 4b). In the probe

trial following acquisition (Fig. 4c), mutant mice spent significantly less time in the target quadrant than controls during each of the three consecutive 30-s intervals. As both groups of animals displayed identical motor abilities, the prolonged latencies of N-CAM-deficient mice in the water maze task are probably attributable to a cognitive impairment.

Spatial learning as tested in the Morris water maze is associated with hippocampal function²⁸. Highly polysialylated N-CAM has been found in the dentate gyrus and the mossy fibres of the hippocampus¹⁶. This staining was confirmed by our immunocytochemical analysis for PSA using monoclonal antibody 735 (Fig. 3b). Immunoreactivity for PSA in the hippocampus seems to be localized to regions showing postnatal neurogenesis and the formation of new neuronal circuits, a correlation already seen in the olfactory bulb. Loss of N-CAM and PSA from the hippocampus might influence plasticity in this system and lead to impaired learning abilities.

In addition to impairment of their learning abilities, a test developed in our laboratory shows that N-CAM-deficient mice also differ in their explorational behaviour. If control mice are placed in a small open box (8.5 × 12 cm, 5.5 cm high), most of them (>70%) escape to explore their environment within a 3-min interval. In contrast, fewer than 20% of the homozygous mutant mice leave the box, indicating an impairment in their exploratory behaviour not seen in the open field test.

There are two reported examples of targeted gene mutations resulting in behavioural changes, α -calcium-calmodulin-dependent kinase II²⁹ and the *fyn* tyrosine kinase³⁰. We show here that the loss of a totally different molecule leads to a comparable behavioural phenotype and to histological changes in the nervous system. □

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METHODS. The water maze was a circular metal pool (115 cm diameter, 57 cm deep) painted flat black. The water level was 1 cm above the goal platform (14.5 cm diameter) which was visually obscured by the addition of non-toxic black powdered paint. The platform was always located in the south-east quadrant of the pool, placed ~25 cm from the wall. The pool was placed in a test room containing a variety of extra-maze cues. On each trial block the mouse was placed in the water facing the wall from all four compass points surrounding the pool, with the order of release determined randomly. A trial ended when either the mouse had climbed onto the hidden platform or 60 s had elapsed, at which point it was removed from the water and placed on the platform. Each mouse spent 10 s on the platform at the end of each trial. The inter-trial interval was ~5 min, the interval separating blocks was 1 h. Following acquisition, each mouse received a single probe trial, where the platform was removed and the mouse was allowed to swim freely for 90 s. Probe trials were videorecorded by a camera centred above the pool. Each videorecord was processed by a video motion analyser (Videomex V, Columbus Instruments). In the open field test the mice were allowed to explore a small empty chamber for 5 min. The field was divided into five sectors; line crosses per min were determined using a video motion analyser.

Conservation of wingless patterning functions in the short-germ embryos of *Tribolium castaneum*

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DURING embryogenesis, all insects reach a conserved, or phylogenetic, stage at which all future segments are present^{1,2}. Different insects, however, arrive at this stage by overtly different pathways. In the long-germ insect *Drosophila melanogaster*, segmentation of the entire embryo occurs nearly simultaneously and results from the action of a cascade of transcriptional regulatory factors that operate in the acellular environment of the syncytial blastoderm^{3,4}. In short-germ insects, segmentation occurs in an anterior-to-posterior sequence, within a cellular environment¹, and might then be dependent on intercellular signalling^{5,6}. To compare the molecular mechanisms of segmentation, we have isolated a homologue of the *Drosophila wingless* gene, a mediator of cell-cell communications⁷⁻⁹, from the short-germ beetle *Tribolium castaneum*. The principal features of *wingless* expression patterns in *Drosophila* are conserved in *Tribolium*, including its early deployment in rostral and caudal domains in the blastoderm, its segmental iteration in cells immediately anterior to cells expressing the *engrailed* gene, and its later restriction to a ventral sector of the developing appendages.

The *Drosophila wingless* (*wg*) gene is a member of the *wnt* gene family. These genes encode small secreted glycoproteins involved in intercellular communication in diverse developmental processes¹⁰. The *Tribolium wingless* (*wg*) complementary DNA encodes a protein with significant amino-acid similarity to other *wnt*-encoded proteins (Fig. 1). In *Drosophila*, *wg* gene expression is first detected in the presumptive head region and in a caudal ring in the early blastoderm (Fig. 2a)¹¹⁻¹³. The onset of expression in these two domains precedes pair-rule gene expression and is unaffected in pair-rule mutations¹⁴. The earliest *Tribolium wg* gene expression is also detected in the blastoderm stage, first in a stripe in the posterior (excluding the pole; Fig. 2b), then shortly after in the presumptive head lobes (Fig. 2c-f). Comparison of the percentage egg length at which the described *Tribolium* pair-rule stripes are detected (refs 15 and 16, and J. Parrish, S. Brown and R. Denell, manuscript in preparation) with the position of the *wg* head expression shows that the *wg* stripe is anterior to the limits of pair-rule gene stripes, and thus is likely to be independent of pair-rule gene activity. Expression continues in these rostral and caudal regions, and the remaining segmental portion of the embryo develops between them (Fig. 3a-d). The early *wingless* expression domains provide the first molecular evidence that both the head and tail are defined (at least in terms of *wg* gene expression) in the blastoderm of a short-germ insect. Although the most studied aspects of the embryonic phenotype of *Drosophila wg* mutations are the resultant defects in segment polarity, the conservation of these early domains suggests an additional functional role for *wingless* at the embryonic termini in both *Tribolium* and *Drosophila*, independent of its subsequent role in segmentation.

The next phase of *wg* messenger RNA expression in both insects is an elaboration of segmentally reiterated stripes. In *Drosophila*, the stripes of *wg* gene expression are one cell wide¹¹⁻¹³ and respond directly to the activity of the pair-rule genes¹⁴⁻¹⁷. These stripes appear virtually simultaneously, with a slight anterior-to-posterior progression, and the stripes in even-numbered parasegments appear first¹⁴. The timing of the appearance of the *Tribolium wg* stripes is different from that in

Drosophila. The *Tribolium* segment-specific *wg* stripes develop in a direct sequential order, as the germ band elongates (Fig. 3a-d). The first stripe appears in the presumptive mandibular segment and is two to three cells wide, followed by a band of six to eight cells without *wg* expression (Fig. 3a). As the germ band elongates, the pattern of 2-3 cells on, 6-8 cells off, reiterates until there are stripes of *wg* RNA expression in three gnathal, three thoracic, ten abdominal segments and the terminus (Fig. 3d). There is no detectable difference in the onset of expression between even- and odd-numbered segments. Additional stripes are added in the developing head on an independent schedule (Figs 3c, d and 4b). Although the timing of *Tribolium wg* expression differs from that in *Drosophila*, the position of the *wg* stripes is consistent with their regulation by pair-rule genes, as is most

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Tri  .....GKNLFGKIVDKGCRETAFIYAITSAAVTHAIARACSEGS
Dro  RWCNSTRNFSRGKNLFGKIVDRGQRETSTFIYAITSAAVTHSIARACSEGT
101
Tri  IDTCNCETHYKGRPHVSGNGGALAGVRDFEWGCGSDNIGFGFTVSREFV
Dro  IESCTCDYSHQSRSPQANHQAGSVAGVRDWEWGCGSDNIGFGFKFSREFV
151
Tri  DAGERGKTIREKMLHNNNEAGRVHVKDQMRQECCKHGMGSGCTIKTCWMR
Dro  DTGERGRNLRKMLHNNNEAGRAHVQAEHRQECCKHGMGSGCTVKTTCWMR
201
Tri  LPPFRVIGDILLKDRFDGASHVAASGHRNNNNNAHQNRPPKPKLNATISSN
Dro  LANFRVIGDNLKARFDGATRVQVTSNLR.ATNALAPVSPNAAGSNVSGSN
251
Tri  SI.....
Dro  GLIIPQSGLVYGEERMLNDHMPDILLENSHPISKIHHNMPSPNSLPQ
300
Tri  .....HSKRENRRKKHYGFQGLKPFNPEHKPGTKDLVYIEMSPGFCEK
Dro  AGQRGGRNRGRQGRKHNRHYFQLNPNHPEHKPGSKDLVYLEPSPSPFCEK
350
Tri  NPKLGIQGTGRLCNDTSMGVDGCDIMCCGRGYRTOEVVVFERCNCTFWH
Dro  NLRQIGLGTGRLCNETSLGVDGCGGLMCCGRGYRDEVVVVERCACTFWH
400
Tri  CCEVVKCDVCRTRTITHTCV
Dro  CCEVVKCKLCRTKKVIYTCQL
450
```

FIG. 1 Alignment of the amino-acid sequences deduced from the nucleotide sequences of the *T. castaneum wg* cDNA (Tri) and the *D. melanogaster wg* cDNA (Dro)^{8,9}. The open reading frame of the *Tribolium* cDNA was determined by alignment to the *Drosophila* sequence; this alignment suggests that the *Tribolium* cDNA lacks ~300 base pairs (bp) of coding sequence at the 5' end. Gaps inserted for optimal alignment are indicated by dots within the sequence, vertical dashes indicate identical amino acids, two dots mark conservative amino-acid substitutions and a single dot marks semi-conservative substitutions. The overall protein similarity deduced from the partial *Tribolium* cDNA to the corresponding segment of the *Drosophila* sequence is 78%, with 66% identical amino acids. Note that all 21 cysteine residues present (underlined) are conserved and that the hydrophilic region absent from the mouse *wnt-1* gene⁸ (amino acids 300-356 in the *Drosophila* sequence) is also absent from *Tribolium*. When the region spanning the first 160 amino acids of the predicted *Tribolium wg* protein was compared with other *wnt*-encoded protein sequences available in the sequence databases, the strongest match was found with the *Drosophila wingless-1* predicted protein sequence, which is 71% similar within this region. The mouse and *Xenopus wnt-1* proteins are 63% and 60% similar, and the other *Drosophila wnt* family members, *wnt-2* and *wnt-3* are 43% and 55% similar within this domain of the *Tribolium wg* sequence.

METHODS. Using a 1.3-kilobase (kb) *EcoRI-HindIII* fragment from the third and fourth exons of a *Drosophila wg* cDNA, three identical clones were isolated from a screen of 1×10^6 bacteriophage from a *Tribolium* embryonic cDNA library prepared in the UNIZAP vector (Stratagene, provided by S. Brown and R. Denell). One of the cDNAs was sequenced using standard procedures. Sequences were aligned using the GCG Sequence Analysis Software and DNASTar. The complete cDNA is 1,557 bp long, 644 of which constitute 3'-untranslated sequence. The entire sequence will be available in the EMBL and GenBank databases.

FIG. 2 The onset of *wingless* transcription in *Drosophila* and *Tribolium* embryos. *Wg* transcripts in *Drosophila* (a; late stage 5 embryo) and *Tribolium* (b–f) are first detected in the blastoderm, in a region surrounding, but not including, the posterior pole (arrows in a, b) and in the presumptive head region (arrowheads in a, c, d, e and f). All embryos are oriented with anterior to the left. a is a dorsal view; c and d are ventral views; b, e and f are lateral views. c and d; e and f are paired images of the same embryo; the embryos in d and f are stained with diaminophenylindole to visualize the nuclei; the smaller embryonic cells are easily distinguished from the larger extra-embryonic cells. The embryo in b is an early blastoderm stage in which the nuclei are spaced 8–9 μm apart. After the completion of the blastoderm, the presumptive embryonic cells begin to shift posteriorly (c, d; completed in e, f) as they initiate gastrulation and germ-band extension. The same domains of *wg* transcription are seen, but now the anterior stripe is in a more posterior position in the egg (arrowhead in e, f). The first segment-polarity stripe (open arrows in e, f) is detected posterior to the initial head stripe. Note that *wg* mRNA is detectable in the anterior tip of the *Drosophila* embryo (a). This region is the presumptive stomodeum^{11–13}. *Wg* mRNA is not detected in the *Tribolium* stomodeum until later in development (Fig. 3d).

METHODS. *Tribolium* eggs were collected from the flour in which the adults were kept with a 400- μm metal sieve. *Tribolium* eggs were dechorionated and devitellinized using *Drosophila* protocols²⁶. Fixation and *in-situ* hybridizations were carried out as in *Drosophila*²⁷, except that the proteinase-K treatment was eliminated. Digoxigenin-labelled RNA probes were made from the *wg* cDNA containing phagemids rescued from the UNIZAP library.

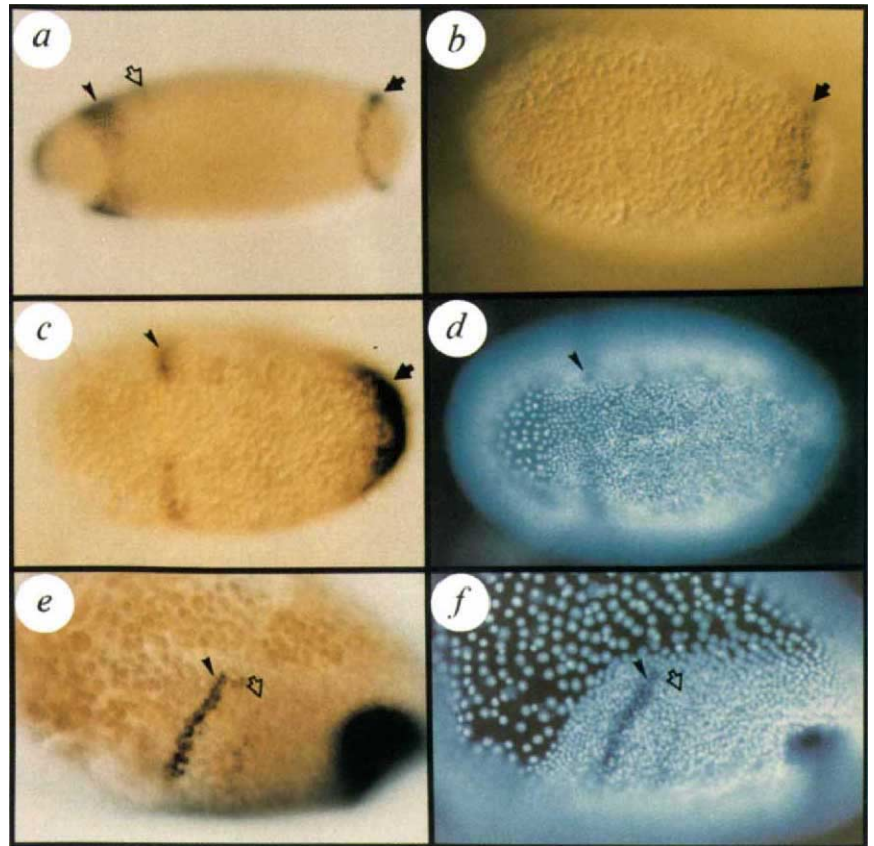
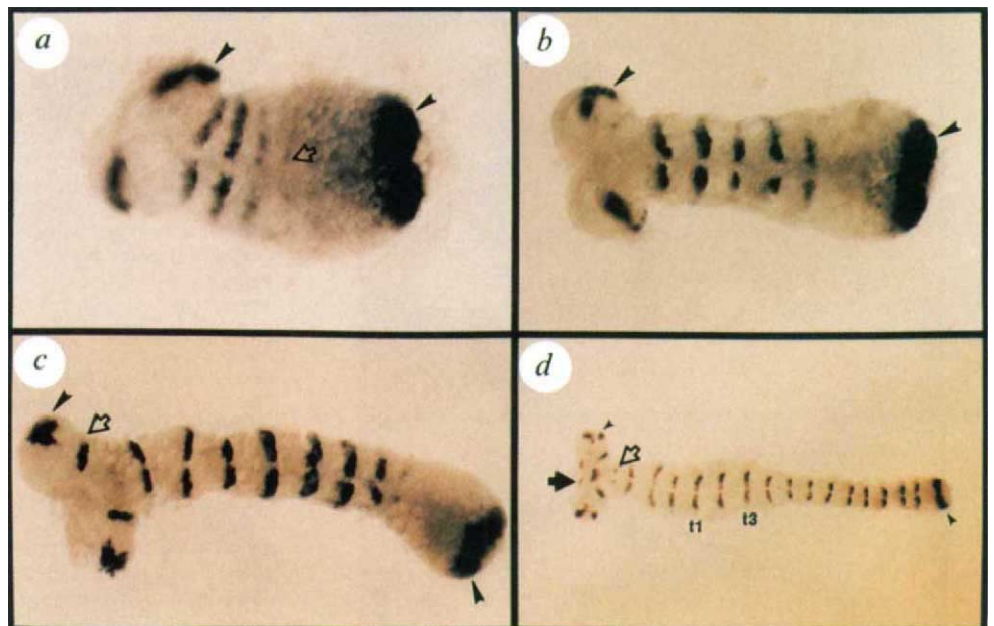


FIG. 3 Development of the *wingless* transcription pattern in *Tribolium*. Segment-specific *wg* stripes appear sequentially, as the germ band elongates (a–d). Each subsequent stripe is two to three cells wide and maintains this width throughout germ-band elongation. (Note that within a *Drosophila* segment, in both the blastoderm and during mid-embryogenesis, the *wg* transcript stripe is only one cell wide^{2,13}.) Each *wg* stripe initiates near the mesoderm-ectoderm boundary (open arrow in a) and spreads dorsally. The gnathal and trunk segments are added in a direct anteroposterior sequence, while additional, more anterior stripes of *wg* expression are added independently. The antennal stripe (open arrow in c) appears after there are five trunk stripes, the intercalary stripe (open arrow in d) appears somewhat later, and finally expression is detected in the labral lobes (solid arrow) and surrounding the stomodeum. The rostral and caudal *wg* stripes initiated earlier are maintained throughout germ-band extension (arrowheads in a–d; the embryo in d is nearly, but not completely, elongated). The development of the *wg*-expression pattern differs from both the elaboration of the *Tribolium* hairy expression pattern¹⁴ and the grasshopper engrailed pattern²⁸. Rostral and caudal *wg* stripes appear first, followed by the stripe in the mandibular segment; additional trunk and head segment stripes are added both anteriorly and posteriorly. In the grasshopper, the prothor-



acic segment is the first to initiate *engrailed* staining, and additional trunk and head segments are added from a prothoracic focus²⁸. In *Tribolium* the 'differentiation centre' of Seidel¹ might then localize to the mandibular segment. All photos represent ventral views of the embryo with anterior to the left. t1, First thoracic segment; t2, second thoracic segment. Methods are as described for Fig. 2.

clearly shown by the spatial relationships of the *Tribolium eve* (ref. 16, and J. Parrish, S. Brown and R. Denell, manuscript in preparation), *en* (refs 15, 16) and *wg* patterns. Thus, there is likely to be a conserved interaction between the pair-rule genes and *wg*, but the specific details of this regulation may be altered between *Tribolium* and *Drosophila*.

Once established in a segment-specific pattern, the *Drosophila* *wg* gene functions in maintaining the polarity of each segment¹⁸. *wg*-expressing cells reside directly anterior to *en*-expressing cells¹³; the boundary between *wg* and *en* corresponds to the parasegment border. Segment polarity is thought to be maintained by intercellular communication between the *wg*- and *en*-expressing cells¹⁸. The spatial relationship of *wg*-expressing cells residing anterior to *en*-expressing cells is conserved in *Tribolium* (Fig. 4a). Thus, the mechanism by which segment polarity is maintained within a segment may also be conserved.

The circumferential stripes of *Drosophila* *wg* expression are later interrupted along the dorsoventral axis, leaving a ventral-medial stripe and a dorsal patch of expression^{11,12}. The *Drosophila* leg imaginal disks form from small clusters of cells which surround the lateral tip of the ventral-medial *wg* stripe¹⁹, and require *wg* function for their formation²⁰ and specification of dorsal/ventral polarity^{21,22}. It is not until the imaginal disks have

grown considerably, however, that *wg* gene expression can be unambiguously mapped to the anteroventral sector of the disk²². In contrast, *Tribolium* exhibits the more ancestral mode of development, forming thoracic legs during embryogenesis, at which time the restriction of *wg* expression to a ventral portion of each segment can be detected (Fig. 4b, c). Most of these *wg*-expressing ventral cells will form part of the appendages. In the antennal, gnathal and thoracic segments, *wg* expression extends from the ventral midline of the germ band to the tip of the developing appendages (Fig. 4b, c).

The conserved *Tribolium* *wg* expression patterns suggest that *wg* serves similar functions in both insects, as do the expression patterns of the other *Tribolium* homologues of the *Drosophila* gap, pair-rule, segment polarity and homeotic genes^{15-17,23}. However, the expression patterns of the *Tribolium* segmentation gene homologues appear in an anterior-posterior progression, rather than simultaneously as in *Drosophila*, consistent with the sequential formation of segments in *Tribolium*. Thus, it could be argued that the difference between long- and short-germ segmentation could be achieved by heterochrony, or a simple change in timing of gene expression. However, there are differences in the early blastoderm expression patterns of the segmentation gene homologues that suggest that the molecular mechanisms operating to create the pattern in the posterior blastoderm of *Tribolium* are

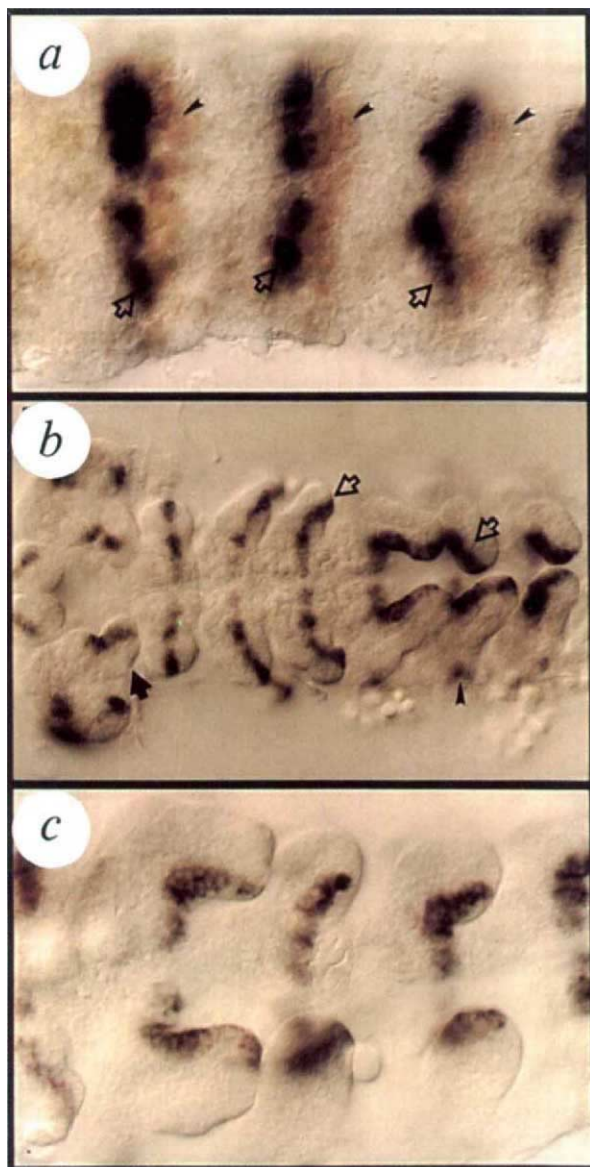


FIG. 4 Conservation of *wg* segment-polarity and limb-patterning functions. *Wg* mRNA (black; arrows) is restricted to those cells anterior to the cells expressing Engrailed protein (brown; arrowheads) (a). The relationship of *wg/en* is seen in the antennal, intercalary, mandibular, maxillary, labial, and thoracic and abdominal segments (1–10); a high magnification of the seventh to tenth abdominal segments is shown. *wg* and *en* expression in the head lobes are not linked; *wg* transcripts are detected in two small head patches and surrounding the presumptive stomodaeum while *en* protein expression anterior to the antennal lobes is seen only in one small area located ventral to the *wg* patch (not shown). Similarly, the most caudal cells expressing *wg* are not bounded by *en*-expressing cells (not shown). This difference in the *wg/en* relationship in the terminal *wg* stripe is consistent with this *wg* domain functioning in the termini independently of the segmented portion of the body. *wg* mRNA is also found in a proximal-distal stripe along the anteroventral margin of the developing appendages (b, c; solid arrow, antennal segment; open arrows, gnathal and thoracic segments; c is a higher magnification of a slightly younger embryo than b), and in patches in the dorsolateral ectoderm (arrowhead, b). These two expression domains are also part of the *Drosophila* *wg* expression pattern. The conserved dorsolateral regulation of the *wg* stripe suggests that elements of the *Drosophila* dorsal-ventral patterning system may also be conserved in *Tribolium*.

METHODS. After labelling with digoxigenin-labelled *wg* RNA probes (see Fig. 2), embryos were washed extensively, dehydrated and stored overnight or longer in 100% ethanol. The *Drosophila* monoclonal antibody Inv 4D9, which detects the Engrailed antigen in many species²⁹, was applied and detected using a biotinylated secondary antibody and the horseradish peroxidase ABC Elite Kit (Vector).

more divergent. For example, expression of the *Drosophila* gap gene *Krüppel* (*Kr*) is localized to the central portion of the anterior-posterior axis of the blastoderm, and is thought to specify thoracic and abdominal fates^{24,25}. Posterior to the *Kr* expression domain are the domains of several additional gap genes and hairy stripes 5, 6 and 7, which specify the remaining abdominal segments^{3,4}. Beyond these is the posterior domain of *wg*, marking cells that will assume a terminal fate. In contrast, the *Tribolium* *Kr* homologue expression has been reported to cover the posterior 20% of the blastoderm^{15,24}, a domain that includes the caudal *wg*-expressing cells (Fig. 2b). Thus, there seems to be an overlap of *Kr* and *wg* expression in the posterior of the early *Tribolium* blastoderm not seen in *Drosophila*. Later in *Tribolium*

development, *Kr* expression coincides with the same thoracic and abdominal segments as it does in *Drosophila*¹⁵. If *Kr* and *wg* expression correlate, respectively, with thoracic/abdominal and terminal fates, then either *Kr* or *wg* expression must be transient in certain cells in the posterior of the blastoderm as the germ band elongates. Whether the larger cell field which forms the entire abdomen arises from the activity of a posterior growth zone or by cell rearrangements is unknown. Nor is it known at what point the blastoderm cells become determined to a particular segmental fate. These differences underscore the need for more detailed blastoderm fate maps and analysis of cell proliferation and movements during germ band elongation in short-germ insects. □

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Amiloride-sensitive epithelial Na⁺ channel is made of three homologous subunits

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THE amiloride-sensitive epithelial sodium channel constitutes the rate-limiting step for sodium reabsorption in epithelial cells that line the distal part of the renal tubule, the distal colon, the duct of several exocrine glands, and the lung. The activity of this channel is upregulated by vasopressin and aldosterone, hormones involved in the maintenance of sodium balance, blood volume and blood pressure^{1,2}. We have identified the primary structure of the α -subunit of the rat epithelial sodium channel by expression cloning in *Xenopus laevis* oocytes³. An identical subunit has recently been reported⁴. Here we identify two other subunits (β and γ) by functional complementation of the α -subunit of the rat epithelial Na⁺ channel. The ion-selective permeability, the gating properties and the pharmacological profile of the channel formed by coexpressing the three subunits in oocytes are similar to that of the native channel.

By functional complementation with the α -subunit of rat epithelial Na⁺ channel (α -rENaC), we were able to isolate two independent complementary DNAs, β -rENaC and γ -rENaC; β -rENaC or γ -rENaC alone were unable to induce amiloride-

sensitive currents (Fig. 1a). Only when cRNAs encoding either β -rENaC or γ -rENaC were coinjected with α -rENaC cRNA was a 3–5-fold potentiation in current observed. When all three (α , β and γ) subunits were expressed together, there was >100-fold potentiation over α -rENaC alone. We conclude that α -rENaC is sufficient to induce channel activity, whereas β - and γ -subunits allow maximal expression of active sodium channels. We have observed, however, that β and/or γ cRNAs can induce small, amiloride-sensitive channel currents, but these currents would represent <1% of the total maximal current observed after coexpression of the three subunits.

The properties of the macroscopic currents expressed in oocytes injected with cRNA encoding α -, β - and γ -subunits are shown in Fig. 1b. A large amiloride-sensitive inward current was observed in the presence of 100 mM Na⁺ which was twice as large in the presence of 100 mM Li⁺. No significant inward current was detected in the presence of 100 mM K⁺. The data indicate that the channel is highly selective to sodium and lithium but is not permeable to potassium. The current-voltage relationship of amiloride-sensitive currents is shown in Fig. 1c from oocytes that had been preincubated in the presence of an external buffer leading to a high intracellular sodium concentration (about 60 mM). In the presence of external sodium or lithium, there was a large inward-rectifying current with a reversal potential close to 0 mV, but in the presence of external potassium, only an outward-rectifying current could be measured, consistent with the outwardly directed sodium gradient created by this experimental condition. When intracellular sodium loading was prevented by incubating the oocytes in an external buffer having a high concentration of K⁺ and a low concentration of Na⁺ (Fig. 1d), the predicted reversal potential was at +50 mV, as would be expected from an intracellular sodium ion concentration ([Na⁺]_i) of ~5 mM. As shown in Fig. 1e, the K_m for sodium activation was 5 mM for Na⁺ and 17 mM for Li⁺, both values being lower than that measured for the current through the native channel¹. The Li⁺/Na⁺ current ratio is similar in both cases. Finally, the K_i values for amiloride (104 nM) and for

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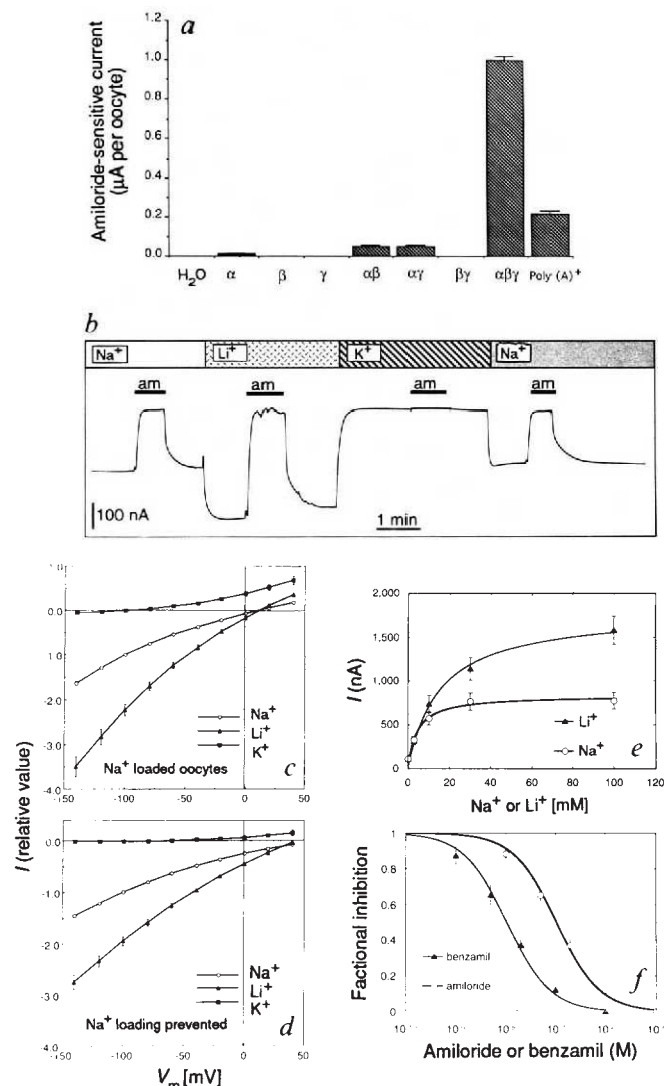
benzamil, an amiloride analogue (11 nM) (Fig. 1f), do not differ from that for the native channel¹.

Single channel recordings were performed by the patch-clamp technique on oocytes injected with α -, β - and γ -subunits. Figure 2a shows typical unitary channel currents from a patch containing two channels using the cell-attached configuration. The channels were recognized by the small inward current at zero membrane potential and by their slow kinetics, with long periods of opening and closing that are characteristic of the epithelial sodium channel¹. The single channel conductance at hyperpolarized potential with 110 mM Na⁺ or Li⁺ in the pipette was 4.6 pS and 7.7 pS respectively. These values compare well with the conductances for the highly selective sodium channel in the apical membrane of amphibian kidney cell⁵ or that of mammalian cortical collecting tubule cells⁶. The current-voltage relationships of unitary currents in the presence of sodium or lithium

in the pipette (Fig. 2b) parallel those of the Li⁺ and Na⁺ macroscopic currents recorded from whole oocytes (Fig. 1d). Unlike the neuronal, tetrodotoxin-sensitive sodium channel⁷, the epithelial sodium channel does not show a strong voltage-dependent gating^{1,8}. The open probability of the epithelial sodium channel is slightly increased by membrane hyperpolarization (Fig. 2c), as reported for the rat kidney epithelial sodium channel⁶.

The amino-acid sequences of β -rENaC and γ -rENaC are shown in Fig. 3a and are compared with that of α -rENaC. The β -rENaC 2,534-nucleotide clone contains a 1,914-nucleotide open reading frame that codes for a 638-amino-acid protein with a predicted M_r of 72,077. The γ -rENaC 3,111-nucleotide clone contains a 1,974-nucleotide open reading frame that codes for a 650-amino-acid protein with a predicted M_r of 74,920. As shown in Fig. 4, the β - and γ -subunit cDNAs hybridized to transcripts

FIG. 1 Macroscopic sodium channel currents recorded in *X. laevis* oocytes injected with the three α , β , γ channel subunits. **a**, Amiloride-sensitive currents in *Xenopus* oocytes injected with H₂O, cRNA (0.01 ng) of α , β and γ -rENaC alone, or combinations of $\alpha\beta$, $\alpha\gamma$, $\beta\gamma$, $\alpha\beta\gamma$ or poly(A)⁺ RNA from the enriched fraction of rat distal colon. **b**, Original recording of amiloride-sensitive current in Na⁺, Li⁺ and K⁺ solutions. The effect of 5 μ M amiloride (am) was tested in the presence of 100 mM Na⁺, Li⁺ or K⁺ at a holding potential of -100 mV. **c** and **d**, The current-voltage relationships of the amiloride-sensitive currents were recorded in Na⁺-loaded oocytes (**c**) and in oocytes in which Na⁺ loading was prevented (**d**) by incubation in a low-Na⁺ buffer. Currents are normalized to the value recorded in the Na⁺-containing solution at -100 mV (**a**, -428 ± 76 nA, $n=9$; and **b**, -563 ± 92 nA, $n=8$). In the presence of Na⁺ or Li⁺, a large inward amiloride-sensitive current was observed, while almost no inward current could be detected with K⁺. When oocytes expressing the Na⁺ channel were incubated in a Na⁺-containing buffer (**c**), their intracellular Na⁺ concentration increased to about 60 mM, as determined from the reversal potential of the amiloride-sensitive Na⁺ current. When Na⁺ loading was prevented by incubation in a low-Na⁺ buffer (**d**), [Na⁺] concentration was ~5 mM, as determined by fitting the Goldman-Hodgkin-Katz equation to the amiloride-sensitive Na⁺ current. Outward currents were proportional to the estimated intracellular Na⁺ concentrations. **e**, Current-ion concentration relationship for Na⁺ and Li⁺ through the amiloride-sensitive conductance. Mean K_m values determined by fitting the Michaelis-Menten equation were 4.9 ± 0.2 mM ($n=8$) for Na⁺, and 16.6 ± 1.4 mM ($n=9$) for Li⁺. **f**, Dose-inhibition curve of the Na⁺ current by amiloride and benzamil at -100 mV in a Na⁺ (80 mM)-containing solution (mean $\pm$ s.e. of 12 and 5 measurements for amiloride and benzamil, respectively). Inhibition constants were 104 nM for amiloride, and 11 nM for benzamil. **METHODS**. mRNA from the distal colon of rats maintained on a low-salt diet was size-fractionated by sucrose-gradient centrifugation³¹. Double-stranded cDNA was prepared from the 2.5–3.5-kb mRNA fraction. After size fractionation, cDNAs larger than 2 kb were ligated into pSPORT1 (BRL) and electroporated into DH10B cells (BRL), yielding ~250,000 clones. *In vitro* transcribed cRNA was prepared from pools of ~20,000 clones, injected into oocytes and assayed for amiloride-sensitive currents by two-electrode voltage clamp³³. The first round of screening revealed no amiloride-sensitive current, but when the screening was repeated in the presence of α -rENaC cRNA, one pool gave larger amiloride-sensitive currents than α -rENaC alone. Amiloride-sensitive currents increased with the reduction of pool size to 1,000 clones. Further subdivisions resulted in the loss of signal. The positive pool containing 1,000 clones was divided in ten and assayed in different permutations until two independent clones, β - and γ -rENaC, were isolated. Oocytes were injected with ~0.01 ng of α , β or γ -rENaC cRNA; 50 ng poly(A)⁺ from rat colon or 50 nl H₂O. Oocytes were clamped at -100 mV and amiloride-sensitive current amplitudes were obtained by determining the difference in current before and after addition of 5 μ M amiloride to the bath. Recordings were made at 22–25 °C from oocytes perfused with a bath solution containing (in mM): 100 sodium gluconate, 2 KCl, 1.8 CaCl₂, 10 HEPES pH 7.2. In **b–f**, *Xenopus* oocytes were injected with 0.1 ng cRNA of the α -rENaC, β -rENaC and γ -rENaC in 50 nl water. They were then incubated (**b**, **d**, **e**, **f**) in amphibian Ringer (80 mM Na⁺, 1 mM K⁺), or (**c**) in a low-Na⁺ Ringer (10 mM Na⁺, 70 mM K⁺) to prevent Na⁺ loading. Electrophysiological measurements were taken 24–48 h after cRNA injection. Current-voltage curves were determined by setting



the command potential for 1 s (at 4-s intervals) from a -100 mV holding potential to values ranging from +40 to -140 mV. Steady-state currents (filtered at 20 Hz) were measured 900 ms after the voltage change. The amiloride-sensitive current was determined as the difference between the current measured in the absence of amiloride and with 5 μ M amiloride. The bathing solutions contained the K⁺ channel blockers barium (5 mM) and TEA (10 mM), and had a low chloride concentration (Cl⁻, 21 mM; gluconate replacement) to reduce the background membrane conductance.

of 2.2 kilobases (kb) and 3.2 kb, respectively, on a northern blot containing mRNA isolated from the distal colon, the kidney cortex and medulla and the lung, but not to mRNA from liver or stomach mucosa. As reported previously³, the α -subunit cDNA hybridized to a 3.7-kb transcript (upper panel) and was co-expressed in the same tissues; it remains to be established whether they are coexpressed in the same cell. The β -rENaC and γ -rENaC subunits each share 34–37% identity with the previously characterized α -rENaC, suggesting that all three subunits are derived from a common ancestor gene. The sodium channel subunit sequences showed significant sequence identity with *mec-4*, *deg-1* and *mec-10*, a family of *Caenorhabditis elegans* genes^{9,10} involved in sensory touch transduction and, when mutated, neuronal degeneration. The greatest identity is found in the hydrophobic domains, but the overall structure of the gene is conserved¹⁰, suggesting that it has a similar function. It is not yet known whether *mec-4*, *mec-10* or *deg-1* can constitute a cation channel and, if so, whether a similar oligomeric structure (α , β , γ) would be required for channel activity.

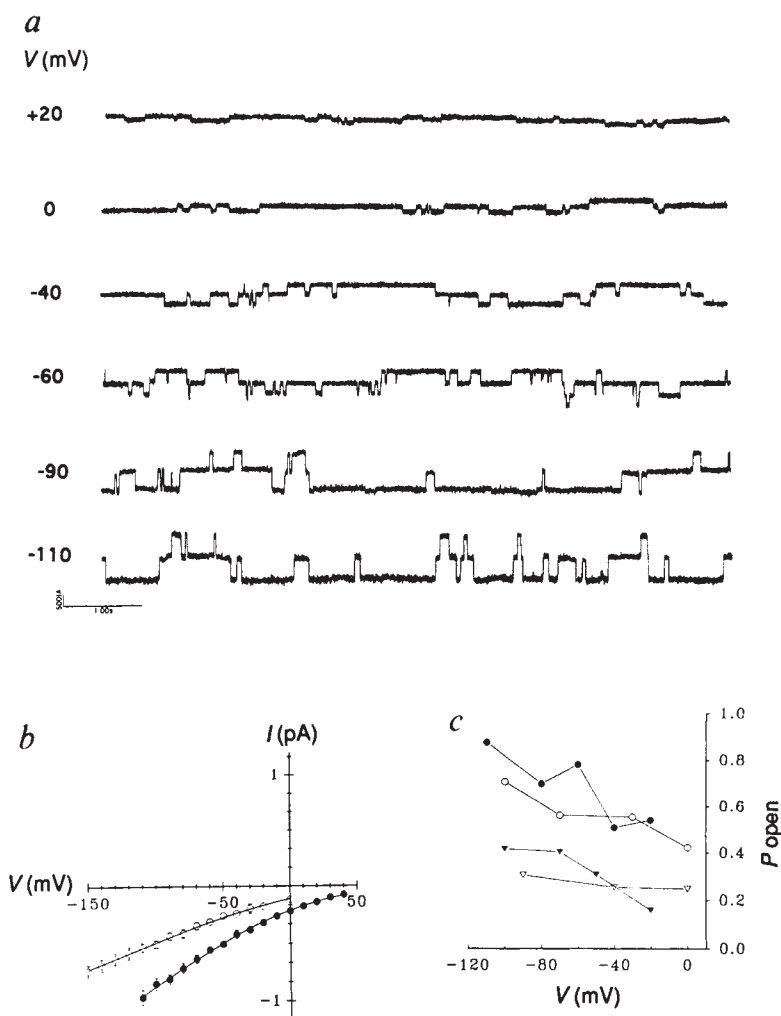
A linear model of the three subunits is shown in Fig. 3b. As none of the subunits expresses a leader sequence, the *N* terminus is predicted to be on the cytoplasmic face. The first putative transmembrane segment (M1) has a predicted α -helical structure and is followed by a stretch of hydrophobic residues (H1) that could adopt a β -sheet structure. A large hydrophilic loop of over 400 residues is predicted to be extracellular. In the case of the α -subunit, this prediction is supported by the fact that four potential *N*-glycosylation sites (N320, N339, N424 and N538) in the extracellular loop are glycosylated¹¹. By analogy, it is likely that the β - and γ -subunit proteins expose the same hydro-

philic domain to the extracellular compartment. The large extracellular loop is followed by a stretch of hydrophobic residues (H2), with a similar hydrophobic index to H1. This is followed by a second putative transmembrane segment (M2) leaving the carboxy-terminal end in the cytoplasmic compartment. The oligomeric structure of the channel is unknown, but we can derive a minimal model of the oligomeric structure of the epithelial sodium channel in which one copy of each subunit is associated in a heterotrimeric protein. By analogy with the voltage-gated potassium channels^{7,12} or the nicotinic receptor¹³, a heterotetrameric (for instance, $\alpha_2\beta\gamma$) or a heteropentameric (for instance, $\alpha_3\beta\gamma$) structure for the epithelial sodium is also conceivable. The relationship between the α -, β - and γ -subunits and the sodium channel protein purified from bovine kidney papilla or from an amphibian kidney cell line¹⁴ remains to be established. Depending on the extent of glycosylation of each of the three subunits, the translation products could give rise to the 95 or 150K polypeptides¹⁵ observed when the native 730K protein complex is analysed under denaturing and reducing conditions^{14,15}.

At present there is no reason to believe that other subunits are required for reconstituting the basic functions of the channel. One cannot, however, exclude the role of associated proteins such as CFTR¹⁶, G protein¹⁷, Apx¹⁸, Abp¹⁹, cytoskeleton proteins²⁰ or endogenous component(s) provided by the oocyte cell, which may well modulate the activity and/or the expression of the channel at the cell surface.

We propose that the rat epithelial sodium channel subunits and the *mec-4*, *mec-10* and *deg-1* proteins all belong to a new gene family encoding cation channels involved in the control of

FIG. 2 Microscopic sodium channel currents recorded in *X. laevis* oocytes co-injected with the three α , β , γ channel subunits. *a*, Unitary Na^+ currents recorded from an oocyte expressing α -, β - and γ -subunits of the epithelial sodium channel. *V* (mV) refers to the intracellular potential with respect to the extracellular face of the patch; the whole oocyte membrane potential was clamped to zero in a high- K^+ medium; pipette contained 110 mM LiCl. Downward deflections represent channel openings. Scale bars, 0.5 pA and 1.00 s. *b*, Current-voltage behaviour of unitary currents recorded with 110 mM Li^+ ($n=6$), or 110 mM Na^+ ($n=8$) as charge carrier in the pipette. The single channel chord conductance between -50 mV and -110 mV was 7.67 pS and 4.58 pS with Li^+ or Na^+ , respectively. Solid lines represent the predictions from the constant field equation: the permeability ratio $P_{\text{Li}}/P_{\text{Na}}$ obtained from the fit was 1.91. *c*, Voltage dependence of single channel open probability (P_{open}) in 4 different cell-attached patches. METHODS. Oocytes were injected with 0.1 ng cRNA of each of three subunits and incubated in a low-sodium medium. Single channel recordings were made in the cell-attached configuration at 22–24 °C. Pipette solution contained (in mM): NaCl 110 or LiCl 110, KCl 2.5, CaCl_2 1.8, HEPES 10, pH 7.4. Bath solution was identical to the pipette solution except that KCl replaced NaCl. Data were sampled at 2 kHz and filtered at 100 Hz for analysis. Single channel current was obtained from gaussian fit of amplitude histograms of at least 50 current transitions. Channel open probability was determined using 50% threshold analysis.



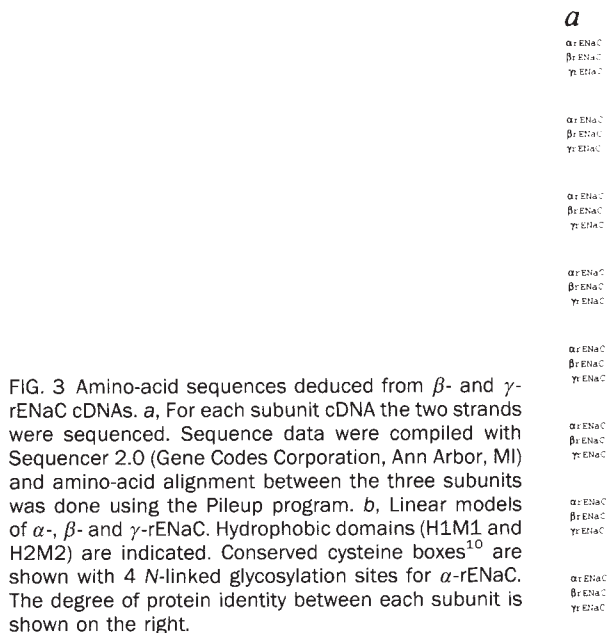


FIG. 3 Amino-acid sequences deduced from β - and γ -rENaC cDNAs. **a**, For each subunit cDNA the two strands were sequenced. Sequence data were compiled with Sequencer 2.0 (Gene Codes Corporation, Ann Arbor, MI) and amino-acid alignment between the three subunits was done using the Pileup program. **b**, Linear models of α -, β - and γ -rENaC. Hydrophobic domains (H1M1 and H2M2) are indicated. Conserved cysteine boxes¹⁰ are shown with 4 N-linked glycosylation sites for α -rENaC. The degree of protein identity between each subunit is shown on the right.

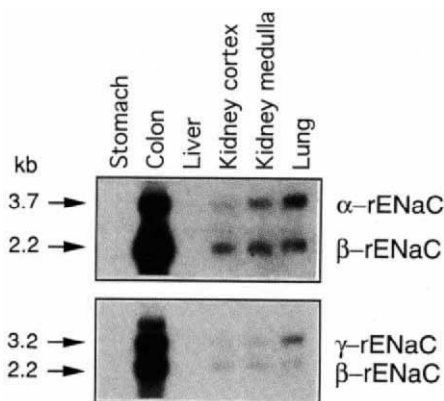
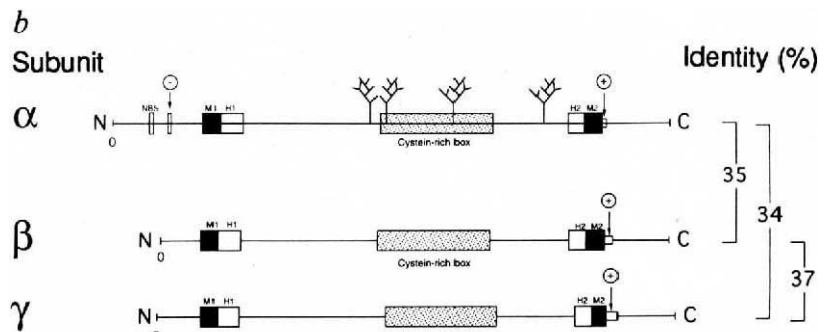


FIG. 4 Northern blot analysis of α -rENaC, β -rENaC and γ -rENaC expression in rat tissues. Molecular mass markers are shown on the left. METHODS. Poly(A)⁺ mRNA (1 μ g) was electrophoresed on a 0.8% denaturing glyoxal agarose gel and transferred to a nylon membrane. The membrane was hybridized with random primed ³²P-labelled dATP full-length rENaC cDNAs. Membranes were first hybridized with α and β probes (upper panel) and then with γ and β probes (lower panel).

the cellular and extracellular volume. This family might also include the mechano-sensitive ion channels in bacterial, yeast and animal cells^{21–25}. Evidence to support this comes from *mec-4*, *mec-10*, *mec-6*, whose gene products are involved in mechanotransduction⁹, the signal transduction of a mechanical stress applied to the cell membrane, which involves the activation of cation channels in a variety of cell types. Examples include mechanical deformation of the cochlear hair cell²⁶, of endothelial cells²⁷, of muscle cells²⁸, stretch of the oocyte membrane²⁹, and osmotically induced cell-volume changes in other cell types³⁰.

In two accompanying papers, the data of Hong and Driscoll³¹ are consistent with the idea that the nematode degenerin (MEC-4) is a subunit of an ion channel, while Huang and Chalfie³² demonstrate the coexpression of two degenerin genes (*mec-4* and *mec-10*) in the same neuron and the requirement of a third gene (*mec-6*) for mechanosensation; this agrees with the hetero-oligomeric model that we propose here for the epithelial sodium channel.

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Gene interactions affecting mechanosensory transduction in *Caenorhabditis elegans*

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GENETIC screening has identified a group of *mec* (mechanosensory) genes that are required for the function of a set of six touch-receptor neurons in the nematode *Caenorhabditis elegans*^{1,2}. Such genes potentially encode components of the mechanosensory apparatus. We have cloned one of these genes, *mec-10*, which is a member of the degenerin gene family (genes such as *mec-4* and *deg-1* (refs 3, 4) that can be mutated to cause neurodegeneration). Because components of an amiloride-sensitive sodium channel (α , β and γ ENaC) from rat^{6–8} share considerable sequence similarity with the *C. elegans* genes, it is likely that degenerins may function as channel proteins. Here we show that two degenerin homologues (*mec-4* and *mec-10*) are expressed in the same cells, although each provides a unique function. Based on genetic data of mutations affecting *mec-10*-induced degeneration, we propose that the products of three genes (*mec-4*, *mec-10* and *mec-6*) form a complex needed for mechanosensation, and that several other *mec* genes may be important in regulating the putative channel complex.

The *mec-10* gene was cloned by homology to conserved sequences in *mec-4* and *deg-1* (refs 3 and 4; these are two degenerins that can mutate to cause the deaths of different neurons) using degenerate oligonucleotides and the polymerase chain reaction (PCR). A full-length *mec-10* complementary DNA encoded an open reading frame (ORF) of 724 amino acids (Fig. 1a) which shared extensive sequence homology with other members of the degenerin family⁵ (*mec-4*, and *deg-1*) and the rENaC genes from rat^{6–8}.

The assignment of this new gene as *mec-10* was suggested by its position on the physical map (the middle of the X chromosome near the gene *kin-9*; A. Coulson, personal communication) and the finding that a *lacZ* fusion with this new gene was

TABLE 1 *mec-10*-induced degenerations at different genetic backgrounds

Gene	Allele (s)	Number of animals	Degeneration (%)
Wild type		109	36
No significant effect			
<i>mec-1</i>	<i>e1066</i>	111	31
<i>mec-2</i>	<i>e1608d</i>	148	35
<i>mec-3</i>	<i>e1338</i>	102	34
<i>mec-5</i>	<i>e1340</i>	122	39
<i>mec-7</i>	<i>u443</i>	112	32
	<i>u278</i>	147	30
<i>mec-8</i>	<i>e398</i>	144	31
<i>mec-9</i>	<i>e1494</i>	105	35
Complete suppression			
<i>mec-3</i>	<i>u467</i>	73	0
<i>mec-4</i>	<i>u253</i>	82	0
	<i>u175</i>	85	0
	<i>u335</i>	83	0
	<i>u339</i>	77	0
<i>mec-6</i>	<i>u450</i>	72	0
Partial suppression			
<i>mec-2</i>	<i>e1084</i>	117	14
	<i>u284sd</i>	215	10
<i>mec-4</i>	<i>u355</i>	147	25
<i>mec-10</i>	<i>u20</i>	143	20
<i>mec-12</i>	<i>e1605</i>	144	10
	<i>u63</i>	158	4
	<i>u241sd</i>	165	20
	<i>e1607</i>	115	15
<i>mec-14</i>	<i>u55</i>	110	6
	<i>u296</i>	146	6
	<i>u82</i>	139	21
<i>mec-15</i>	<i>u75</i>	132	12
Enhancement			
<i>mec-10</i>	<i>e1515</i>	131	56
	<i>e1715</i>	137	53
	<i>u154</i>	109	58
	<i>u332</i>	117	51
	<i>u390</i>	117	56
<i>mec-18</i>	<i>u69</i>	134	49
	<i>u138</i>	127	53
	<i>u182</i>	131	51

For most *mec* genes, especially those that have not been cloned, alleles producing the strongest mutant phenotype^{1,2} were used. All the mutations are recessive unless otherwise indicated. The *mec-3(u467)* allele contains a suppressible amber mutation early in the *mec-3* coding region (ref. 15, and M.C., unpublished results). The *mec-3(e1338)* allele also has a nonsense mutation¹⁵, but does allow for some expression of a *mec-7* gene in the PLM cells⁹. This difference might account for the lesser effect of *e1338* on *uls2* (even though *e1338* blocked the PLM expression of a *mec-10-lacZ* fusion, see Fig. 2). The sites of the *mec-4* mutations have recently been determined (K. Hong and M. Driscoll, personal communication): *mec-4(u253)* contains a 300-bp deletion which eliminates the first hydrophobic domain, and *mec-4(u175)* and *mec-4(u335)* have nonsense mutations before the first hydrophobic domain—these mutations act as null alleles; *mec-4(u339)* has the identical change found in *mec-10(e1515)* and also produces a null phenotype; *mec-4(u355)*, with a *Tc1* transposon insertion in the coding region³, produces a partial loss-of-function phenotype: animals are partially touch-sensitive as adults, possibly as a result of somatic excision of the transposon or alternative splicing that produces some wild-type MEC-4. The *mec-7(u443)* mutation deletes the gene¹⁶, and the *mec-7(u278)* mutation is a missense allele that causes ectopic touch cell branching (C. Savage et al., manuscript submitted). The *mec-10* mutations are indicated in Fig. 1. *mec-2(e1084)* is an apparent null mutation, as suggested by intra-allelic complementation experiments; *u284* is a semidominant allele of *mec-2* (ref. 1). *e1608* is a dominant allele of *mec-2* and shows positive intraallelic complementation with some other *mec-2* alleles¹, which may be the reason why it differs from other *mec-2* alleles in its effect on *uls2*-caused degeneration. The integrated element *uls2* was crossed into different genetic backgrounds and newly hatched larvae were counted for PLM cell degenerations using differential interference contrast microscopy as described in Fig. 3 legend. For each strain except for those in the 'complete suppression' group, two separate experiments were done using >50 animals in each experiment. The difference between the two experiments was always less than 5%.

fied defects within the *mec-10* coding region in five different *mec-10* strains, thus confirming the identity of this gene (Fig. 1b, c). One of the mutations, *e1515* (S105F), was defective in a highly conserved region just before the first of the two hydrophobic domains. As an identical mutation also eliminates *mec-4* function (K. Hong and M. Driscoll, personal communication), this region appears to be important for the function of both proteins.

The co-localization of two degenerin genes, *mec-4* (ref. 9) and *mec-10*, in the same six touch cells indicates that they might overlap in function (previous genetic experiments with *deg-1* (ref. 4) suggested that this gene was redundant). The fact that mutation of either gene results in a touch-insensitive phenotype^{1,2}, however, implies that they are not redundant genes. To determine whether overexpression of one gene could compensate for the loss of the other, we transformed wild-type *mec-10* and *mec-4* genomic DNA into *mec-4* and *mec-10* mutants, respectively. Although each DNA can rescue the cognate mutant (28 out of 29 transient transformants of *mec-4* were touch-sensitive, and 27 out of 32 for *mec-10*), they could not rescue the other mutant phenotype (84 transient and 26 relatively stable transformants were tested for *mec-4* DNA transforming *mec-10* mutants; 21 transient transformants and three relatively stable lines were tested for *mec-10* DNA transforming *mec-4* mutants), suggesting that each gene provides a unique function for mechanosensation.

In all the dominant degeneration-causing alleles of *mec-4* (ref. 3) and *deg-1* (E. Wolinsky, personal communication), a conserved alanine near the beginning of the second hydrophobic domain has been mutated. To determine whether a similarly mutated *mec-10* could also cause a similar vacuolar degeneration, we transformed wild-type animals with an *in vitro*-mutagenized *mec-10* genomic clone (*mec-10*(A673V)). Touch-cell degenerations were seen in some of the newly hatched larvae of two relatively stable transgenic lines (Fig. 3). (Degeneration was never seen when transformants received wild-type *mec-10* DNA.) To characterize the *mec-10*-induced degenerations better, we generated a strain in which 6–8 intact copies of the injected DNA had integrated (as element *uls2*) into the chromosomal DNA.

Compared with *mec-4*(*e1611*), which causes the death of all six touch cells in over 90% of the animals, *uls2* produced a

weaker phenotype: cell death, usually just of the PLM cells (a few ALM deaths were seen), occurred only at 15 °C and in only 36% of the newly-hatched *uls2* larvae (Table 1). This incomplete penetrance was an advantage because it allowed us to identify mutations that either enhanced or suppressed these deaths in double mutants with alleles of other *mec* genes (Table 1).

Mutations in five *mec-10* alleles increased the frequency of *mec-10*-induced degeneration (to as much as 58%). It is likely that these are at least partial loss-of-function alleles and that the increased frequency of cell deaths resulted from the loss of competition from the wild-type *mec-10* product. We do not know whether this competition is with multiple copies of MEC-10 (the *mec-10* protein) or with another protein. Competition is also indicated by the apparently paradoxical suppression of *uls2* cell death by the *mec-10*(*u20*) mutation. This *trans*-acting effect suggests that *u20*, although recessive, is a gain-of-function allele that corrects the *uls2* defect. We envisage that *uls2* causes a channel to be open for a greater proportion of the time, thus leading to cell lysis; the *u20* product (change of a glycine to arginine at a position three amino acids downstream from the mutated alanine) would counter this effect. Interestingly, mutations affecting the equivalent codon in *deg-1* (changing the glycine to either arginine or glutamate) have been isolated as suppressors of different degeneration-causing mutations in *deg-1*; these suppressors acted in both *trans* and *cis* configurations (E. Wolinsky, personal communication; J. Garcia-Añoveros and M.C., unpublished data). E. Wolinsky (personal communication) first suggested from these results that DEG-1 could form a multimeric complex. Our results suggest that degenerin multimers may generally occur, a model supported by recent experiments with Mec-4 (ref. 11).

As with *mec-4* (and *deg-1*; ref. 4), the *mec-10*-induced degenerations are completely dependent on the wild-type function of the *mec-6* genes (Table 1). However, although *mec-4* deaths can occur without *mec-10* activity⁴, *mec-10* deaths require *mec-4* activity (Table 1). These data, as well as the touch-insensitivity that results from the loss of activity of these genes, suggest a model in which *mec-4*, *mec-10* and *mec-6* all contribute to a membrane complex (channel) which is needed for mechanosensory transduction (perhaps with multiple subunits of some or all of these proteins), and in which *mec-6* and *mec-4* are essential

FIG. 2 β -galactosidase expression of *mec-10-lacZ* in third-stage larvae of a, wild-type, and b, *mec-3*(*e1338*). All six touch-receptor neurons and two FLP and two PVD neurons stain in wild-type animals (in 100 animals, the percentage of cells stained was: FLP/R 90%, ALML/R 89%, AVM 82%, PVM and PVDL/R 57%, PLML/R 92%). No staining was seen in *mec-3*(*e1338*) animals. Although *mec-10* is also expressed in FLP neurons, which are needed for touch sensitivity at the tip of the head²², and the PVD (a pair of neurons that mediate a response to a harsh-touch stimulus¹⁰), no detectable effects of *mec-10* mutations were found on the function of these cells¹⁰ (data not shown).

METHODS. A *mec-10-lacZ* fusion (TU#57) was constructed with *mec-10* genomic DNA, including 2.8 kb of upstream sequence and 2.8 kb of the coding sequence (from exon 1 to the middle of exon 10, which includes sequences encoding the first hydrophobic domain) inserted in-frame upstream of the *E. coli lacZ* gene in the vector pPD34.110 (ref. 23). Transformant lines carrying this *mec-10-lacZ* fusion were generated by germline transformation²⁴ using pRF4 (*rol-6*(*su1006*)) as a marker (100 ng μ l⁻¹ for each DNA). Roller animals were stained for β -galactosidase activity as described²³. More than 50 animals (L3 larvae to adults) were counted. Expression of *mec-10-lacZ* in *mec-3*(*e1338*) animals was examined in transformants obtained by co-injection of pRF4 and TU#57 into the CB1338 strain, and also by crossing the extrachromosomal DNA arrays from a wild-type background into CB1338.

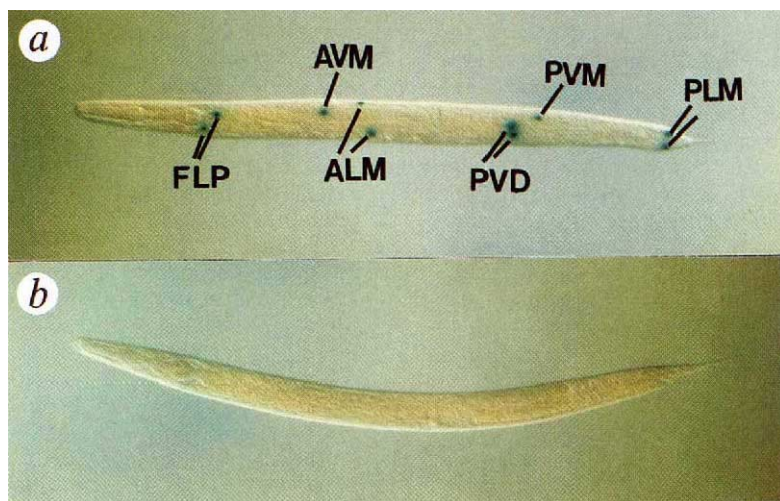




FIG. 3 PLM degeneration caused by *uls2*. One degenerating PLM cell, swollen several times the size of a normal cell, is shown.

METHODS. Plasmid TU#56, which carries an alanine-to-valine change at amino acid 673 of MEC-10 (*mec-10(A673V)*) introduced by *in vitro* mutagenesis using a Muta-Gene kit (Bio-Rad), was used for germline transformation of a *mec-10(e1515)* strain. PLM degenerations were seen in some newly hatched larvae of transgenic animals. An integrated strain was isolated from the progeny of 127 F_1 animals after γ -ray irradiation of one relatively stable line as described⁹. The integrated DNA element, *uls2*, is likely to be near a chromosomal end as it did not map near any centrally positioned *dpy* genes on the six *C. elegans* chromosomes. The amount of both intact and total *mec-10(A673V)* DNA that had been integrated was estimated by probing restriction-enzyme-digested genomic DNA from the wild-type and the integrated strain with a 2.6-kb *HindIII* genomic DNA fragment of *mec-10* and comparing the intensity of bands of expected sizes on Southern blots. The intact genomic clone of *mec-10* should be contained in a 8.5-kb *Clal* fragment. A 2.6-kb *HindIII* band was used to quantify the total amount of *mec-10* coding sequences. The intensity of the Southern blot was quantified using a Molecular Dynamics phosphorimager. The number of intact *mec-10* copies is 6 to 8, though the total number of *mec-10*-containing sequences is about 400. Degenerations were observed in animals that had been grown at 15 °C for at least two generations. Newly hatched larvae (within 3 to 4 h of hatching at 15 °C) were collected and examined using differential interference contrast microscopy.

for the formation of the channel complex at the membrane but *mec-10* is not. In this model, without either *mec-4* or *mec-6* the channel cannot form, and thus mutated *mec-10* alone cannot increase membrane conductance or cause degeneration. In the absence of wild-type *mec-10* product, the channel complex can still form (although it does not function normally as the animals are touch-insensitive) and toxic *mec-4* can disrupt membrane conductance and result in degeneration. Our data are consistent with those of Canessa *et al.*⁸, which suggest that three degenerin-like proteins contribute to the formation of the amiloride-sensitive sodium channel in rats.

Mutations in several other *mec* genes (*mec-2*, *mec-12*, *mec-14*, and *mec-15*) partially suppressed the *mec-10*-induced degenerations and those in *mec-18* enhanced them. The molecular mechanisms by which these genes function are not understood. The mammalian amiloride-sensitive sodium channel consists of several different proteins¹² and some of the subunits, such as Abp¹³ and Apx¹⁴, may have modulatory effects on the channel activity. It is possible that these *C. elegans* genes may also encode products that are important in regulating the putative mechanosensory channel complex. □

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A transmembrane domain of the putative channel subunit MEC-4 influences mechanotransduction and neurodegeneration in *C. elegans*

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ABERRANT ion channel activity plays a causative role in several human disorders^{1–3}. Inappropriately regulated channel activity also appears to be the basis for neurodegeneration induced by dominant mutations of *Caenorhabditis elegans mec-4* (*mec-4(d)*), a member of the degenerin gene family postulated to encode a subunit of a mechanosensory channel⁴. The degenerin gene family has been defined by two *C. elegans* genes, *mec-4* and *deg-1* (ref. 5), which can mutate to gain-of-function alleles that induce degeneration of specific groups of neurons. A related mammalian gene, rat α -rENaC, induces an amiloride-sensitive Na^+ current when introduced to *Xenopus* oocytes⁶, strongly suggesting that degenerin genes encode ion channel proteins. Deduced amino-acid sequences of the degenerins include two predicted membrane-spanning domains^{6,7}. Here we show that conserved amino acids within the second membrane-spanning domain (MSDII) are critical for MEC-4 activity and that specific substitutions within MSDII, whether encoded in *cis* or in *trans* to a *mec-4(d)* mutation, block or delay the onset of degeneration. Remarkably, MSDII from two other family members, *C. elegans deg-1* (ref. 5) and rat α -rENaC (ref. 6), can functionally substitute for MEC-4 MSDII in chimeric proteins. Our results support a structural model for a mechanosensory channel in which multiple MEC-4 subunits are oriented such that MSDII lines the channel pore, and a neurodegeneration model in which aberrant ion flow through this channel is a key event.

In the degenerins, the carboxy-terminal hydrophobic domain MSDII is characterized by highly conserved amino acids that align on one face of the predicted transmembrane α -helix (Fig. 1a, b). On the basis of structural similarities to characterized ion channels^{8,9}, we propose that MSDII lines the ion channel pore and that conserved amino acids project into the channel lumen to influence ion transport.

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TABLE 1 Functional assay of *mec-4* alleles encoding substitutions in MSDII

a				
Plasmid	Allele	Mutation introduced	Amino-acid substitution	% Touch-sensitive in <i>mec-4(u253)</i>
pRF4	<i>rol-6(su1006)</i>	—	—	0
TU#12	<i>mec-4(+)</i>	—	—	38
ZB#10	<i>mec-4(bz1)</i>	CTT to GCC at 4,514–4,516	L450A	0
ZB#11	<i>mec-4(bz2)</i>	CTT to AAA at 4,514–4,516	L450K	0
ZB#12	<i>mec-4(bz3)</i>	TGG to GCA at 4,517–4,519	W451A	0
ZB#13	<i>mec-4(bz4)</i>	T to C at 4,517	W451R	0
ZB#14	<i>mec-4(bz5)</i>	G to C at 4,524	G453A	0
ZB#15	<i>mec-4(bz6)</i>	G to A at 4,523	G453R	0
ZB#16	<i>mec-4(bz7)</i>	T to A at 4,529	S455T	0
ZB#17	<i>mec-4(bz8)</i>	A to T at 4,538	T458S	2
ZB#18	<i>mec-4(bz9)</i>	A to C at 4,549	E461D	0
ZB#19	<i>mec-4(bz10)</i>	GA to AT at 4,547–4,548	E461I	0
b				
Plasmid	Allele	Chimaeric gene	% Touch-sensitive in <i>mec-4(u253)</i>	
TU#12	<i>mec-4(+)</i>	<i>mec-4</i>	38	
ZB#20	<i>mec-4(bz11)</i>	<i>mec-4/mMSDIIΔ</i>	0	
ZB#21	<i>mec-4(bz12)</i>	<i>mec-4/mMSDI</i>	0	
ZB#22	<i>mec-4(bz13)</i>	<i>mec-4/dMSDII</i>	34	
ZB#23	<i>mec-4(bz14)</i>	<i>mec-4/rMSDII</i>	41	
ZB#24	<i>mec-4(bz15)</i>	<i>mec-4/rMSDIIS458T</i>	0	

a, Effects of single amino-acid substitutions for conserved residues in MSDII. Synthetic *mec-4* alleles and the amino-acid substitutions they encode are numbered as in ref. 4. Alleles were tested for ability to rescue the loss-of-function allele *mec-4(u253)*, which has a partial deletion of *mec-4* coding sequences (K.H. and M.D., unpublished observations) and renders animals touch-insensitive. Plasmid pRF4, which carries the dominant marker *rol-6(su1006)*¹⁸, was coinjected with *mec-4* alleles to facilitate identification of roller transformants. For each *mec-4* allele tested, at least 100 rollers from three independent transgenic lines were assayed for touch sensitivity as described¹⁰; scores listed are the average values for these three lines. Not all synthetic alleles failed to complement *mec-4(u253)*. Two conservative substitutions introduced, V463L and E468D, did not disrupt MEC-4 function (data not shown). b, MSDII domain swap experiments. Plasmids tested harbour chimaeric *mec-4* genes in which coding sequences for MEC-4 MSDII (amino acids 448–467, indicated by black line in Fig. 1a) were replaced by sequences encoding the domain indicated after the slash: mMSDIIΔ, a deletion of MEC-4 MSDII; mMSDI, another hydrophobic domain that is present in the full length *mec-4* protein (M.D. et al., manuscript in preparation) encoded by nucleotides 1,626–1,688 (ref. 4); dMSDII, amino acids 229–248 from DEG-1 MSDII⁵; rMSDII, amino acids 582–601 from the rat α -rENaC protein⁶; rMSDIIS458T, same as rMSDII except that Thr replaces Ser at the site corresponding to MEC-4(458). All plasmids were coinjected with pRF4. Scores are the average per cent touch sensitivity for a minimum of 100 adult rollers from each of three independent transgenic lines. The *mec-4* alleles encoding substitutions in or for MSDII were constructed according to ref. 19 and introduced into animals as described in ref. 20. To ensure that transforming DNA, which exists as an extrachromosomal array that can be lost during cell division, would be present in as many cells as possible, only rollers from lines in which transforming DNA was passed on to a minimum of 30%, but >58% on average, of progeny were scored. Plasmids that failed to complement *mec-4(u253)* were also introduced into the wild-type background (data not shown). All disrupted touch sensitivity in the *mec-4(+)* background, an effect that is most probably due to production of high levels of mutant protein in transgenic animals harbouring high copy numbers of introduced alleles. The dilution effect supports the argument that alleles tested produce reasonably stable mutant *mec-4* proteins.

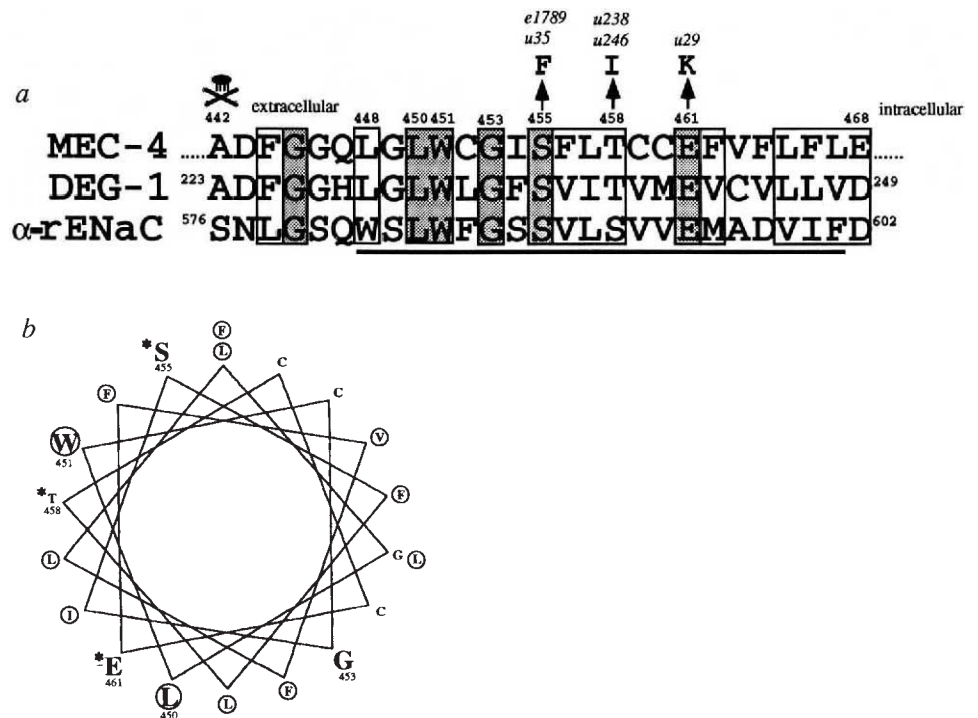
To investigate the function of MSDII, we sequenced the MSDII coding region from 50 ethylmethanesulphonate (EMS)-induced loss-of-function *mec-4* alleles (*mec-4(lf)*)^{10,11}. We identified five missense mutations encoding amino-acid substitutions in this domain (Fig. 1a). These mutations affect the conserved polar serine and threonine residues at positions 455 and 458, respectively (S455, T458), and a negatively charged glutamate at position 461 (E461) which we propose will project into the channel lumen. To investigate the functional requirement for conserved amino acids in MSDII, we used site-directed mutagenesis to construct *mec-4* alleles encoding single amino-acid substitutions and tested their ability to complement *mec-4(u253)*, a loss-of-function mutation that renders animals insensitive to gentle touch (Table 1a). We found that all tested alleles encoding substitutions for the five strictly conserved amino acids, including those encoding conservative substitutions (S455T, T458S, E461D), failed to complement the *mec-4(u253)* mutation. Conserved residues are therefore critical for MEC-4 function and we suggest that the channel pore has precise structural requirements.

Having established that conserved amino acids on one face of MSDII are necessary for MEC-4 activity, we wondered whether any amphipathic α -helix that harboured these residues at the appropriate positions in MSDII might function in the context of the *mec-4* protein. We therefore replaced MEC-4 MSDII with the corresponding 20-amino acid domain from either DEG-1 or rat α -rENaC. Chimaeric genes were tested for their ability to confer touch sensitivity when introduced into *mec-4(u253)* ani-

mals (Table 1b). Although alleles encoding a deletion in MEC-4 MSDII or encoding a second copy of MEC-4 MSDI instead of MSDII do not rescue the *mec-4(u253)* mutation, hybrid genes encoding either DEG-1 or α -rENaC MSDII in place of MEC-4 MSDII can complement this loss-of-function mutation. This is remarkable because, although MSDII is one of the best conserved domains within the degenerin family, it has only 25% identity and 70% similarity amongst the three sequenced family members⁷ (Fig. 1a). Interestingly, introduction of a single amino-acid change within the MSDII domain of the *mec-4/rMSDII* chimaera (*mec-4/rMSDIIS458T*), designed to make the conserved face more similar to that of MEC-4 MSDII, inactivates the chimaera. Thus, the intact transmembrane domain appears to be required for channel activity. We conclude that MSDII is essential for MEC-4 function and that this domain is functionally conserved among family members assayed. Our domain-swapping experiments support the claim that the *C. elegans* degenerins and the mammalian α -rENaC function in similar ways in different cell types.

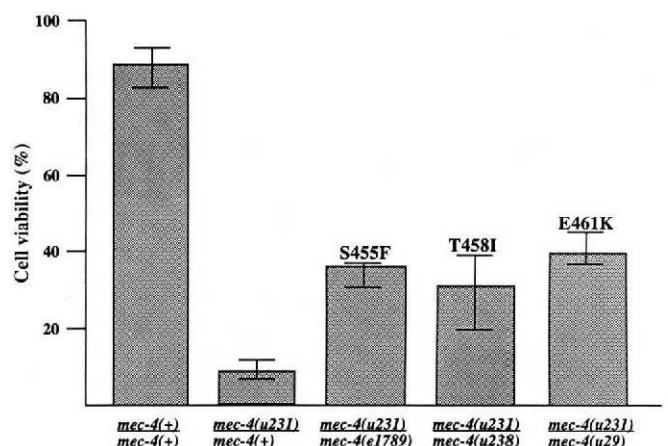
Degeneration of six *C. elegans* mechanosensory neurons is induced by gain-of-function *mec-4(d)* alleles that encode substitutions of large-side-chain amino acids adjacent to MSDII at MEC-4 position 442 (ref. 4). We have previously postulated that, in the MEC-4(d) channel, steric hindrance around the side chain at position 442 prevents the channel from closing properly, resulting in excess ion flux through the channel and consequent neurodegeneration^{4,12}. If so, an amino-acid substitution in

FIG. 1 MSDII is amphipathic, conserved among degenerins and includes amino acids required for MEC-4 function. *a*, Alignment of MSDII and adjacent regions of MEC-4, DEG-1 and α -rENaC. MEC-4 amino acids are numbered as in ref. 4. Corresponding amino-acid numbers in *C. elegans* DEG-1 (ref. 5) and rat α -rENaC⁶ are listed. The predicted MSDII is indicated by the black line. MEC-4 amino acid position 442, the site at which substitutions of large-side-chain amino acids induce neurodegeneration, is indicated by a skull-and-crossbones icon. Amino acids identical in all three degenerins are boxed in grey; conserved residues are boxed in white. Topography is as predicted in ref. 12. Amino-acid substitutions encoded by EMS-induced recessive *mec-4* mutations are indicated by arrows; names of alleles encoding the depicted substitutions are in italics. Identified mutations are: e1789 and u35, C to T at nucleotide 4,530; u238 and u246, C to T at nucleotide 4,539; u29, G to A at nucleotide 4,547. *b*, Helical wheel plot of MEC-4 MSDII. Amino acids 448–467 are represented by the single-letter code and are numbered as in *a*. Hydrophobic residues are circled. Amino acids identical in MEC-4, DEG-1 and α -rENaC are in large bold typeface and amino acids affected by EMS-induced mutations are marked with an asterisk. METHODS. Computational analysis was performed using the GCG package²¹. Sequences encoding MEC-4 amino acids 437 to 497 were determined from 50 EMS-induced recessive *mec-4* alleles described in refs 10 and 11. Genomic DNAs were amplified using sense primer 5'-ACAGGCGTTCGTTGACTATTCATG-3' and antisense primer



5'-TATTTTAAGACACAACTTGCAAT-3'. A second round of asymmetric polymerase chain reaction (PCR) using either primer was used to generate the template for DNA sequence analysis. Sequencing primers were sense 5'-AATGCCGAATTACTACTAGCGAC-3' or the antisense primer above. DNA was sequenced according to the manufacturer's specifications (US Biochemical) except that 1 μ l dimethylsulphoxide was added to each labelling reaction. In the cases of the five alleles that harboured missense mutations, nucleotide changes were verified by sequencing the complementary strand.

FIG. 2 Single amino-acid substitutions in MSDII can partially suppress *mec-4(d)*-induced degeneration in *trans* heterozygotes. Histogram depicts the per cent PLM cells surviving in heterozygotes containing the death-inducing *mec-4(d)* allele u231 and a second *mec-4* allele that encodes a single amino-acid substitution within MSDII (e1789, u238, u29). *mec-4* genotypes are listed below the x-axis; the amino-acid change encoded by the *mec-4* allele being tested for suppression in *trans* is shown above each bar. All heterozygotes were also *jeln1*+/+; *dpy-17*/+. *jeln1* contains an integrated concatamer of pRF4 [rol-6(*su1006*)] and pNW115 (which includes a *mec-7/lacZ* fusion gene expressed primarily in the touch receptors¹³). Cell survival was assayed by scoring for β -galactosidase activity in PLM cells of egg-bearing adults. Values indicated are the average of at least three trials in which a minimum of 68, but almost always greater than 120, animals were scored. Error bars indicate minimum and maximum scores in these trials. Strains homozygous for *jeln1*; *mec-4*(+) or *jeln1*; *mec-4*(u231) exhibited 90% and 4% PLM staining, respectively. Partial death suppression is not likely to result from fewer active monomeric MEC-4 channels present per cell in *mec-4*(u231)/*mec-4*(r) as compared with *mec-4*(u231)/*mec-4*(+) heterozygotes since animals heterozygous for *mec-4*(d) and a *mec-4* amber allele, *mec-4*(u72) (K.H. and M.D., unpublished data) do not exhibit suppression (3% staining). *mec-4*(u35) and *mec-4*(u246) act similarly to their isoalleles *mec-4*(e1789) and *mec-4*(u238) in this assay (data not shown). It should be noted that the efficiencies of suppression for a given substitution in *trans* are not necessarily directly comparable to efficiencies of degeneration of the corresponding *cis* configuration presented in Table 2. In the *cis* experiments of Table 2, *mec-4* alleles are present in high copy number and only one form of the *mec-4* protein is present in the touch cells whereas in the *trans* experiments presented here, cells have the normal *mec-4* gene dosage but have two different types of MEC-4 subunits. The different channel composition expected in these two experiments could explain why substitutions at positions 455 and 458 block degeneration



and substitutions at position 461 delay degeneration in the *cis* experiment of Table 2, whereas these substitutions act equally effectively in *trans* to suppress degeneration. Note also that, in the absence of the A442V substitution, all three substitutions in MSDII disrupt normal MEC-4 activity.

METHODS. Genetic manipulations were at 20 °C as in ref. 22. Alleles used were: LGI, *jeln1*¹³; LGIII, *dpy-17*(e164)²²; LGX, *mec-4*(e1789, u29, u35, u72, u231, u238, u246)^{10,11}. *mec-4* heterozygotes were constructed by mating *mec-4* males to strain ZB22 (*jeln1*; *dpy-17*(e164); *mec-4*(u231)). Staining for β -galactosidase activity was as in ref. 23. Non-Dpy Rol cross progeny were examined at $\times 400$ magnification for staining of PLM cells. Staining of one or both PLM cells was considered positive.

TABLE 2 Effects of single amino-acid changes in MSDII on neurodegeneration caused by the MEC-4 A442V substitution

Plasmid	Combination of introduced alleles	Amino-acid substitutions	% PLM cell death	
			L1	L4
pRF4	<i>rol-6(su1006)</i>	—	0	0
TU#12	<i>mec-4(+)</i>	—	0	2
TU#14	<i>mec-4(u231)</i>	A442V	54	3
ZB#25	<i>u231 bz1</i>	A442V L450A	0	0
ZB#26	<i>u231 bz2</i>	A442V L450K	0	0
ZB#27	<i>u231 bz3</i>	A442V W451A	0	0
ZB#28	<i>u231 bz5</i>	A442V G453A	0	0
ZB#29	<i>u231 bz6</i>	A442V G453R	0	0
ZB#30	<i>u231 e1789</i>	A442V S455F	0	8
ZB#31	<i>u231 bz7</i>	A442V S455T	0	0
ZB#32	<i>u231 u238</i>	A442V T458I	0	3
ZB#33	<i>u231 bz8</i>	A442V T458S	0	0
ZB#34	<i>u231 u29</i>	A442V E461K	0	50
ZB#35	<i>u231 bz9</i>	A442V E461D	0	38
ZB#36	<i>u231 bz10</i>	A442V E461I	0	45

mec-4 alleles encoding both A442V, which in the context of an otherwise normal MEC-4 protein induces degeneration of six touch receptor neurons⁴, and a second amino-acid substitution in MSDII were introduced with pRF4 into *mec-4(u253)*. Transgenic lines were examined during the first larval stage (L1) and the fourth larval stage (L4) for swollen degenerating PLM neurons in the tail. Death in the L1 stage was scored by examining a population of ~200 L1 animals from each of three transgenic lines derived for each construct. In the L1 stage, Rol animals harbouring introduced DNA cannot be distinguished from those that have lost transforming DNA, so only a fraction of the L1 population is expected to exhibit degenerations. Degeneration in the L4 stage was scored by examining at least 50 Rol animals from each of the three lines. Scores listed are the average per cent degeneration from three independent lines. For comparison we note that in wild-type animals we do not detect degenerations in L1 or L4 animals ($n > 200$); in the *mec-4(u231)* strain, which has two genomic copies of the allele encoding the A442V substitution, 99% of the animals show degenerations in the L1 stage but there are no animals showing degenerations in the L4 stage as the touch receptors lyse by this time ($n > 200$). Site-directed mutagenesis and generation of transgenic animals were performed as described for experiments in Table 1 except that plasmid TU#14 (ref. 4), which harbours *mec-4(u231)*, was used to generate template DNA. The specific nucleotide changes introduced to encode the listed substitutions are as in Table 1. Allelic combinations for the doubly substituted alleles are listed to facilitate cross-reference to Fig. 1a and Table 1a. Actual designations for *mec-4* alleles in plasmids ZB25–36 are *bz16–bz27*. Observations were made at $\times 400$ magnification using Nomarski differential interference contrast optics. Animals that had at least one swollen PLM cell were scored as positive. Transgenic lines scored segregated transforming DNA to progeny a minimum of 30%, and on average 58%, of the time.

MSDII that disrupts channel conductance could be expected to block degeneration. We therefore constructed *mec-4* alleles encoding both A442V, which by itself induces neurodegeneration, and a second substitution within MSDII. Doubly substituted alleles were introduced into the *mec-4(u253)* background and transgenic lines were examined for degeneration of two touch receptors in the tail, the PLM cells (Table 2). We find that amino-acid substitutions for conserved amino acids in MSDII alter degeneration to markedly different degrees: some substitutions block degeneration altogether whereas others merely delay the onset of degeneration. This suggests that ion uptake is required for degeneration to occur and that the onset of degeneration is related to how effectively the channel transports ions. As certain substitutions disrupt touch sensitivity but only delay degeneration (for example, compare data in Tables 1a and 2 for substitutions at 461), it seems that mechanosensory transduction requires precisely regulated ion flow whereas degeneration requires a cumulative influx of ions. The MEC-4

channel will need to be electrophysiologically characterized to verify these hypotheses.

Many ion channels are multimeric and some include more than one subunit of a given type. Genetic evidence suggests that DEG-1–DEG-1 interactions occur (E. Wolinsky and J. Garcia-Anoveros and M. Chalfie, personal communications). We reasoned that if multiple MEC-4 subunits are included in the functional channel, the effects of MEC-4(d), postulated to increase permeability as a result of inefficient channel closing, might be circumvented by the presence of a second mutant *mec-4* protein harbouring a substitution that interferes with ion flow. To test this, we quantitated cell death in heterozygotes harbouring one *mec-4(d)* allele and one *mec-4* allele encoding a single amino-acid substitution in MSDII. Expression of a *mec-7/lacZ* fusion gene, which is primarily restricted to the touch receptor neurons¹³, was used as a marker for cell viability. Our results (Fig. 2) show that MSDII amino-acid substitutions *in trans* to the degeneration-inducing amino-acid substitution A442V can partially suppress cell death. This suppression suggests that one MEC-4 subunit can influence the activity of another and that more than one MEC-4 subunit is included in the functional channel complex.

The rat epithelial amiloride-sensitive sodium channel includes at least three distinct but homologous subunits¹⁴. The MEC-4 channel is likely to have a similar make-up, including the homologous MEC-10 degenerin coexpressed in the touch receptor neurons as a subunit¹⁵. Genetic evidence is consistent with the hypothesis that the *mec-6* gene, required for degeneration induced by *mec-4(d)*⁵, could encode a third subunit necessary for the complex to function.

Mechanosensitive ion channels mediate cellular responses to stretch, contraction and osmotic changes in prokaryotic, plant and animal cells^{16,17}. Despite their ubiquitous distribution and participation in cellular activities, mechanosensory channels remain the only major class of channels for which there is no definitive structural information. Our model for the structure of a candidate mechanosensory channel in *C. elegans* touch receptor neurons places MEC-4, which includes a domain forming the channel pore and a domain that influences channel closing, in a key role. Our domain-swap experiments suggest that biochemical and electrophysiological tests of this model using either the nematode or mammalian family members will advance understanding of the function of this newly discovered class of ion channels. □

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Regulation of chemotaxis by the platelet-derived growth factor receptor- β

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CHEMOTAXIS is an important component of wound healing, development, immunity and metastasis, yet the signalling pathways that mediate chemotaxis are poorly understood. Platelet-derived growth factor (PDGF) acts both as a mitogen and a chemoattractant¹. Upon stimulation, the tyrosine kinase PDGF receptor- β (PDGFR- β) autophosphorylates² and forms a complex that includes SH2(Src homology 2)-domain-containing proteins such as the phosphatidylinositol-specific phospholipase C- γ (ref. 3), Ras-GTPase-activating protein (GAP)⁴, and phosphatidylinositol-3-OH kinase⁵. Specific tyrosine-to-phenylalanine substitutions in the PDGFR- β can prevent binding of one SH2-domain-containing protein without affecting binding of other receptor-associated proteins^{6,7}. Here we use phospholipase C- γ (ref. 8) and PDGFR- β mutants⁹⁻¹¹ to map specific tyrosines involved in both positive and negative regulation of chemotaxis towards the PDGF-BB homodimer. Our results indicate that a delicate balance of migration-promoting (phospholipase C- γ and phosphatidylinositol-3-OH kinase) and migration-suppressing (GAP) activities are recruited by the PDGFR- β to drive chemotaxis towards PDGF-BB.

To determine whether receptor autophosphorylation is necessary for migration towards PDGF-BB, we used CHO cells trans-

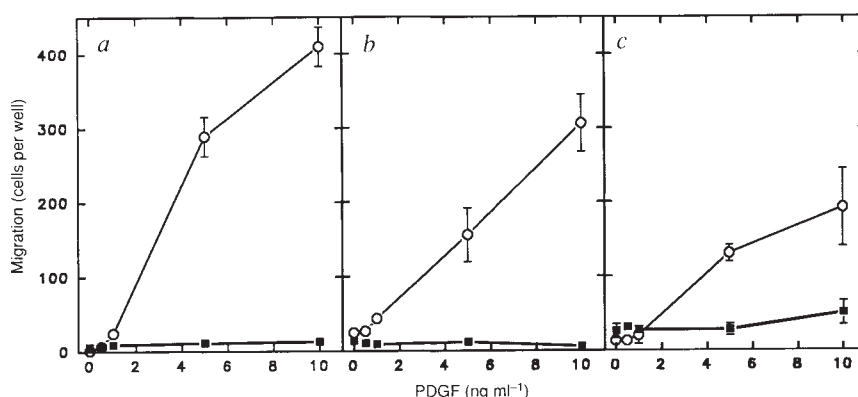
fecting with wild-type or mutant PDGFR- β ¹⁰. CHO cells transfected with normal murine PDGFR- β subunits¹⁰ migrated in a dose-dependent manner towards a PDGF-BB gradient (Fig. 1a) in a multiwell Boyden chemotaxis chamber¹². In the absence of PDGF-BB there was a low level of random migration across the filter. CHO cells transfected with an autophosphorylation-negative PDGFR- β ¹¹ (lysine 602 substitution to alanine) failed to migrate (Fig. 1a). We also tested canine kidney epithelial cells (TRMP cells¹³) expressing wild-type (WT) or mutant human PDGFR- β subunits. All of the TRMP cell lines expressed similar numbers of wild-type or mutant PDGFR- β ⁹ and showed a small amount of random migration, as did CHO cell lines. TRMP(WT) cells expressing wild-type human receptor also migrated towards PDGF-BB, whereas TRMP(R635) cells (expressing PDGFR- β with arginine at position 635) having kinase-inactive PDGFR- β subunits failed to migrate towards PDGF (Fig. 1b). Thus, autophosphorylation of PDGFR- β subunits promotes migration towards PDGF-BB.

We subsequently tested the role of specific PDGFR- β tyrosines in mediating chemotaxis towards PDGF-BB. PDGFR- β tyrosine residue 1,021 specifically binds phospholipase C- γ (refs 9, 14, 15). Although products of phospholipase C (PLC) activation have been postulated to mediate chemotaxis, PLC itself has not been studied directly. The γ -isozyme of PLC contains SH2 domains that mediate binding to activated tyrosine kinases¹⁶. TRMP(F1021) cells express PDGFR- β subunits with a tyrosine-to-phenylalanine substitution at position 1,021 which fail to bind or activate PLC- γ in response to PDGF, yet bind and activate other signalling molecules such as phosphatidylinositol-3-OH kinase⁹. TRMP(F1021) cells showed reduced migration towards PDGF (Fig. 1c), indicating that tyrosine 1,021 positively regulates migration towards PDGF-BB. Similar results were seen in CHO cells expressing mutant murine PDGFR- β (our unpublished results).

NIH3T3 cell lines were used to evaluate further the role of PLC- γ in migration towards PDGF-BB. PLC- γ tyrosines 771 and 783 were previously identified as sites phosphorylated by activated PDGF receptor⁸. Y783F cells express a non-functional PLC- γ (tyrosine 783 changed to phenylalanine) mutant in addition to their endogenous PLC- γ . These cells showed wild-type PLC- γ activity in response to PDGF and their migration towards PDGF was unaffected (Fig. 2a). 2B2 cells, which overexpress wild-type PLC- γ , have PDGF-inducible PLC- γ activity that is 5-fold higher than in non-transfected cells⁸. However, the

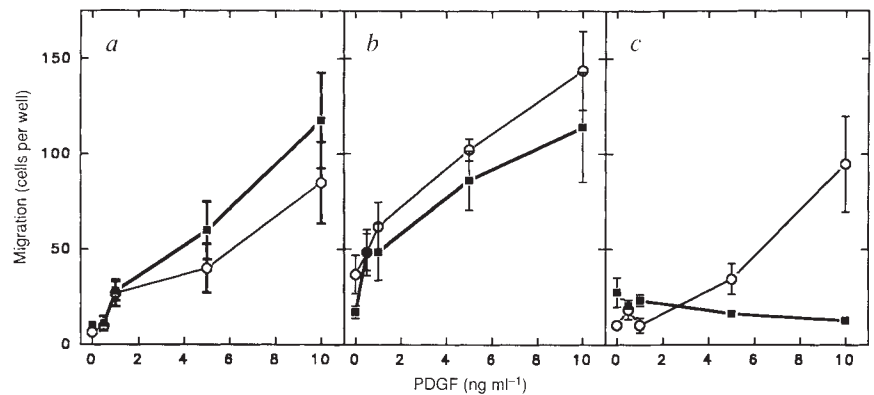
FIG. 1 Migration towards PDGF-BB of cells expressing wild-type human or murine PDGFR- β or with mutant receptors deficient in autophosphorylation or in binding PLC- γ . **a**, Migration towards PDGF-BB of CHO-R18 cells (○) expressing wild-type murine PDGFR- β , and of CHO-602 cells (■) expressing kinase-inactive murine PDGFR- β . **b**, Migration towards PDGF-BB of TRMP(WT) cells (○) expressing wild-type human PDGFR- β , and of TRMP(R635) cells (■) expressing kinase-inactive human PDGFR- β . **c**, Migration towards PDGF-BB of TRMP(WT) cells (○) and TRMP(F1021) cells (■) expressing PLC- γ -association- and activation-defective human PDGFR- β .

METHODS. Migration was assayed using a modified Boyden chamber assay¹². 25 × 80 mm 8- μ m polyvinylpyrrolidone-free filters (nucleopore) were coated for one or two days with 100 μ g ml⁻¹ collagen type I (Collaborative Research) in 0.2 M acetic acid. A dry, coated filter was placed on a blind 48-well chamber (Neuroprobe) containing PDGF-BB diluted in Dubelco Modified Eagle's Medium (DMEM) (Gibco). The gasket and the top well piece were then screwed in place. After trypsinization and dilution, 15,000 CHO cells or 30,000 TRMP cells in 50 μ l DMEM were added to the top wells. The chamber was



incubated at 37 °C in 5% CO₂ for 4 h, then disassembled and the cells scraped off the filter. The migrating cells were then fixed in formalin, washed in PBS, and stained overnight in Gills triple-strength haematoxylin (Polysciences). After three washes in water, the filter was mounted in glycerol. All cells in an area representing a well were counted visually. Error bars represent the standard error of three or four replicates.

FIG. 2 Migration of NIH3T3 cells transfected with PLC- γ and mutant PLC- γ towards PDGF-BB. *a*, Migration towards PDGF-BB of NIH3T3 cells ($\circ$) and Y783F cells ($\blacksquare$) expressing inactive PLC- γ in addition to endogenous PLC- γ . *b*, Migration towards PDGF-BB of Y783F cells ($\circ$) and 2B2 cells ($\blacksquare$) overexpressing wild-type PLC- γ . *c*, Migration towards PDGF-BB of NIH3T3 cells ($\circ$) and Y771/783F cells ($\blacksquare$) expressing Y771F and Y783F substituted PLC- γ which suppresses endogenous PDGF-inducible PLC- γ activity. Assays are described in Fig. 1 legend.



migration of 2B2 cells towards PDGF was also unaltered (Fig. 2b). Thus, wild-type PLC- γ activity may achieve a threshold for migration towards PDGF that is unaffected by either excess non-functional protein or excess wild-type PLC- γ .

Y771/783F cells express a mutant PLC- γ , with tyrosines 771 and 783 both substituted to phenylalanine, which does not associate with activated PDGF receptor but does suppress by 60% the endogenous PLC activity normally observed after PDGF stimulation⁸. Y771/783F cells showed reduced migration towards PDGF-BB (Fig. 2c) but random motility was normal. These results with PLC- γ or PDGFR- β mutants suggest that PLC- γ activity promotes migration towards PDGF-BB.

The PDGFR- β kinase region is interrupted by a kinase-insert domain¹⁷; PLC- γ binds outside this kinase insert domain. CHO cells expressing murine PDGFR- β with the kinase insert domain deleted at residues 684–765 (Δ Ki) failed to migrate towards PDGF-BB (Fig. 3), even though receptor expression in the Δ Ki cells is higher than in wild-type PDGFR- β -transfected cells¹⁰. The Δ Ki PDGFR- β fails to associate with GAP or phosphatidylinositol-3-OH kinase¹¹ yet can autophosphorylate and activate PLC- γ ¹⁰. Thus, PLC- γ activation is not sufficient for PDGF-BB-induced chemotaxis. Kinase insert domain function is needed for mitogenesis¹⁰ and chemotaxis.

The kinase insert domain contains tyrosines involved in binding GAP and phosphatidylinositol-3-OH kinase¹¹. When expressed in CHO cells, murine PDGFR- β having both Y708F and Y719F substitutions fails to bind phosphatidylinositol-3-OH kinase after PDGF activation, although GAP and PLC- γ are bound⁶. Figure 4a shows that, in contrast to CHO-R18 cells expressing wild-type PDGFR- β , CHO cells expressing a PDGFR- β incapable of binding phosphatidylinositol-3-OH kinase (CHO(Y708/719F) cells) do not migrate towards PDGF. Results were similar in TRMP cells expressing human PDGFR- β mutants (Fig. 4b). Thus, PDGFR- β tyrosines involved in binding phosphatidylinositol-3-OH kinase promote migration towards PDGF-BB.

In addition to phosphatidylinositol-3-OH kinase, GAP also binds the kinase insert domain of PDGFR- β . Murine PDGFR- β with a Y739F substitution expressed in CHO cells fails to bind or phosphorylate GAP upon activation, but still binds phosphatidylinositol-3-OH kinase and PLC- γ (ref. 6). Figure 4c shows that CHO(Y739F) cells, which express a PDGFR- β that cannot bind GAP, migrate in greater numbers toward PDGF-BB than do CHO cells expressing wild-type receptor (CHO-R18 cells). Similar results were observed with TRMP cells expressing mutant human PDGFR- β (Fig. 4d). Thus, the PDGFR- β tyrosine responsible for GAP association and phosphorylation negatively regulates migration towards PDGF-BB.

We next tested whether the positive or negative tyrosine regulatory effect is dominant. We used TRMP cells expressing PDGFR- β subunits with tyrosines 740, 751 and 771 substituted by phenylalanine (TRMP(F740/751/771) cells). This receptor is

unable to bind either GAP or phosphatidylinositol-3-OH kinase specifically⁷. TRMP(F740/751/771) cells showed a diminished chemotactic response to PDGF-BB relative to cells expressing wild-type receptors (Fig. 4e). However, both cell types showed similar unstimulated random motility. Thus, as predicted from experiments with Δ Ki cells, positive regulation due to the human PDGFR- β tyrosines at 740 and 751 that bind phosphatidylinositol-3-OH kinase dominates over the negative regulation attributable to the tyrosine at 771 that binds GAP. These experiments and assays using F1021 cells show that neither PLC- γ nor phosphatidylinositol-3-OH kinase binding to PDGFR- β is sufficient for migration towards PDGF-BB. Apparently, PLC- γ and phosphatidylinositol-3-OH kinase cooperate to promote migration towards PDGF-BB.

Cell locomotion requires an intricate balance of movement-promoting and movement-suppressing events. Signals within a cell must be spatially regulated, turning on or off a signal at the leading edge of a cell while performing the opposite action at the trailing edge of a cell. We have shown that these opposing activities are initiated at the receptor level. We describe positive and negative regulation of chemotaxis by PDGFR- β tyrosines responsible for recruiting secondary signalling molecules. We cannot exclude the possibility that tyrosines involved in binding PLC- γ , phosphatidylinositol-3-OH kinase or GAP associate with as-yet unidentified molecules. However, PLC- γ and phosphatidylinositol-3-OH kinase activation modulate actin assembly^{18,19}. Thus, two molecules may cooperate to regulate a

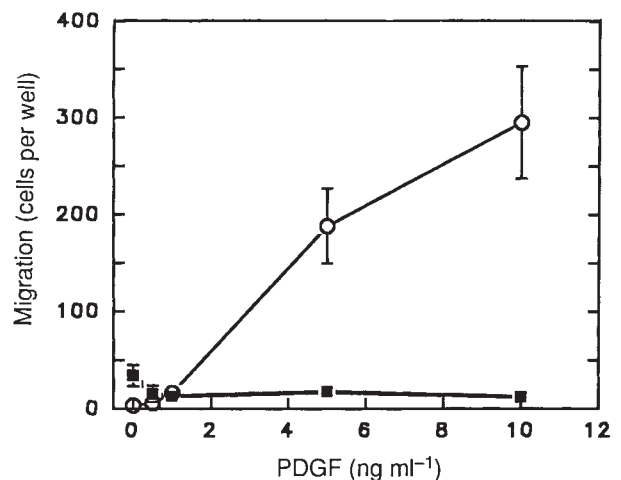


FIG. 3 Migration towards PDGF-BB of CHO-R18 cells transfected with murine wild-type PDGFR- β ($\circ$) or with Δ Ki mutant ($\blacksquare$) murine PDGFR- β . Assays are described in Fig. 1 legend.

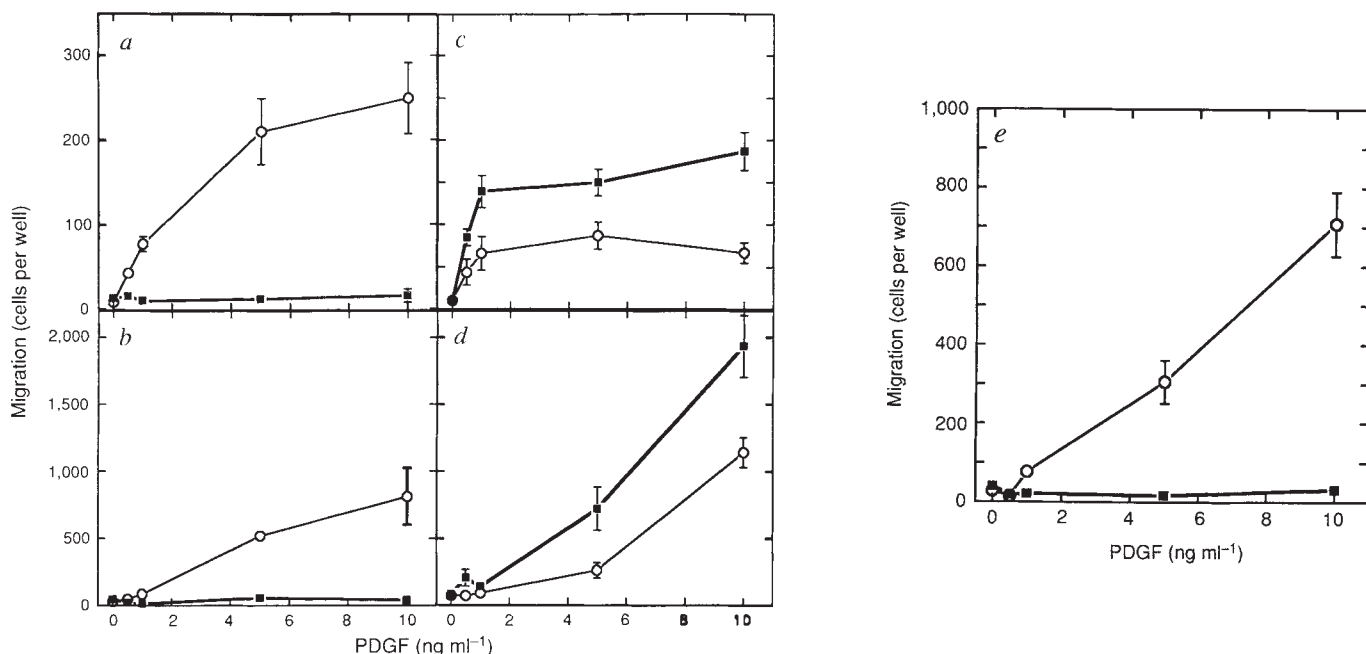


FIG. 4 Migration towards PDGF-BB of cells expressing wild-type PDGFR- β or mutant receptors defective in binding phosphatidylinositol-3-OH (PtdIns-3-OH) kinase and/or GAP. *a*, Migration towards PDGF-BB of CHO-R18 cells ($\circ$), and of CHO(Y708/719F) cells ($\blacksquare$) expressing Y708F and Y719F substituted PDGFR- β , which fail to bind PtdIns-3-OH kinase after PDGF activation but still bind GAP and PLC- γ (ref. 6). *b*, Migration towards PDGF-BB of TRMP(WT) cells ($\circ$), and of TRMP(F740/751) cells ($\blacksquare$) expressing Y740F and Y751F substituted human PDGFR- β , which fail to bind PtdIns-3-OH kinase after PDGF activation but still bind GAP and PLC- γ (ref. 7). *c*, Migration towards PDGF-BB of CHO-R18 cells ($\circ$), and of CHO(Y739F) cells ($\blacksquare$) expressing Y739F substituted murine

PDGFR- β , which fail to bind or phosphorylate GAP upon activation but still bind PtdIns-3-OH kinase and PLC- γ (ref. 6). *d*, Migration towards PDGF of TRMP(WT) cells ($\circ$) and of TRMP(F771) cells ($\blacksquare$) expressing Y771F substituted human PDGFR- β , which fail to bind or phosphorylate GAP after PDGF activation but bind PtdIns-3-OH and PLC- γ (ref. 7). *e*, Migration of TRMP cells expressing wild-type human PDGFR- β or a mutant receptor defective in binding both GAP and PtdIns-3-OH kinase. Migration towards PDGF-BB of TRMP(WT) cells ($\circ$) and TRMP(F740/751/771) cells ($\blacksquare$) expressing human PDGFR- β defective in GAP and PtdIns-3-OH kinase association but still able to bind PLC- γ (ref. 7). Assays are described in Fig. 1 legend.

particular biological activity such as chemotaxis. GAP binding to the PDGFR- β does not affect mitogenesis^{6,7} but does influence chemotaxis. Therefore, a single protein that associates with the PDGFR- β may modulate one biological activity and not another. Our results indicate that PLC- γ and phosphatidylinositol-3-OH kinase association with activated PDGFR- β promotes migration, whereas GAP association with activated PDGFR- β suppresses migration toward PDGF-BB. In a cell expressing wild-type PDGFR- β , the net effect is migration towards PDGF-BB. $\square$

Modulation of gene expression by calreticulin binding to the glucocorticoid receptor

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CALRETICULIN is a multifunctional protein that acts as a major Ca^{2+} -binding (storage) protein in the lumen of the endoplasmic reticulum¹. It is also found in the nucleus², suggesting that it may have a role in transcription regulation. Calreticulin has been reported to bind to the synthetic peptide KLGFFKR³, which is almost identical to an amino-acid sequence in the DNA-binding domain of the superfamily of nuclear receptors⁴⁻⁶. Could calreticulin interact with the DNA-binding domain of these receptors and affect their function? Here we report that the amino terminus of

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Figure 1a shows the amino-acid sequence of the DNA-binding domain of the human glucocorticoid receptor (GR). This region of GR and other steroid receptors (Fig. 1b) contains a conserved amino-acid sequence KxFFKR (consensus sequence KxFF(K/R)R) (Fig. 1b) which is similar to the calreticulin-binding sequence (KLxFFKR) found in a highly conserved motif in the cytoplasmic domain of the α -subunit of integrins³. The crystal structure of the GR DNA-binding domain complexed with DNA has recently been solved, showing that the sequence KxFFKR is in direct contact with the GRE⁷. These observations indicated that calreticulin may interact with the DNA-binding domain of the GR. We therefore expressed the

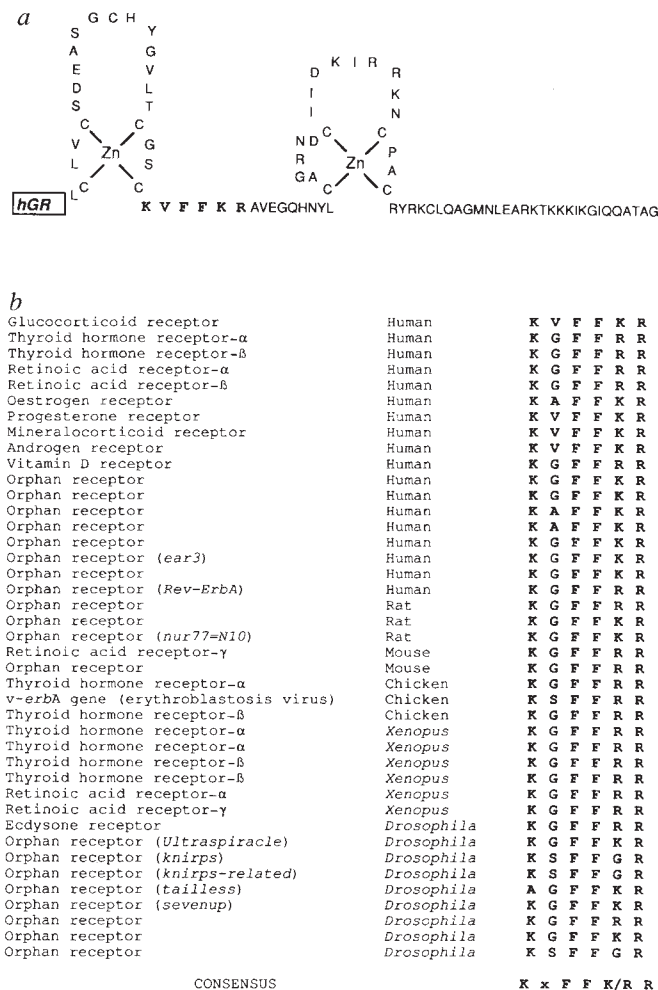


FIG. 1. The DNA-binding domain of the steroid receptors. In *a*, The 86-residue DNA-binding domain of the human glucocorticoid receptor (hGR) is shown in the classical zinc-finger representation. The consensus amino-acid sequence KxFF(K/R)R is found in the DNA-binding domain of hGR (*a*) and other steroid receptors (*b*). The amino-acid sequences in *b* are taken from ref. 6. The conserved residues (KxFF(K/R)R) are probably involved in interactions with calreticulin.

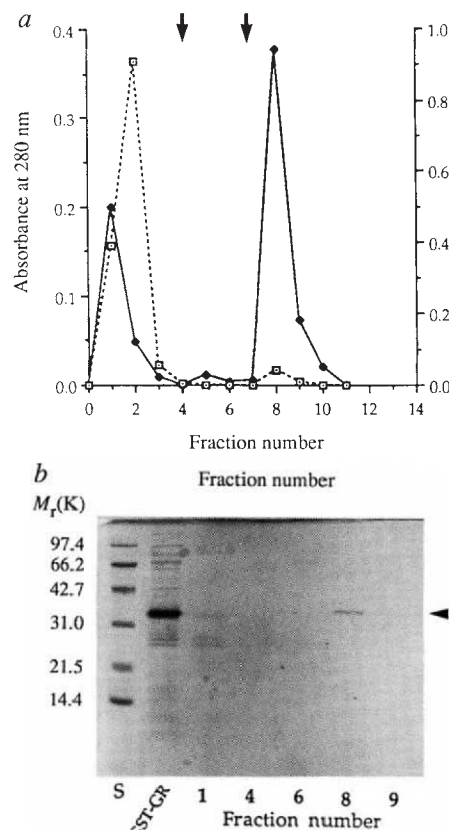


FIG. 2 The GST-GR fusion protein binds specifically to a calreticulin affinity column. *a*, Glutathione-Sepharose-purified GST (2 mg; broken line) of GST-GR (solid line) were applied onto a calreticulin affinity column equilibrated with a buffer containing 100 mM CaCl₂, 2 mM MgCl₂, 0.1 mM CaCl₂ and 40 mM Tris, pH 7.2. The column was then washed with a buffer containing 100 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂ and 40 mM Tris, pH 7.2. This was followed by (left-hand arrow) low-salt buffer (elution 5 mM EDTA, 100 mM NaCl and 40 mM Tris, pH 7.2) and (right-hand arrow) high-salt buffer elution (20 mM EDTA, 750 mM NaCl and 40 mM Tris, pH 7.2). Fractions were analysed by absorbance at 280 nm. *b*, SDS-PAGE of protein fractions from the GST-GR separation in *a*. S, low-range Bio-Rad molecular weight standards. GST-GR refers to glutathione-Sepharose-purified GST-GR fusion protein. Arrowhead indicates GST-GR.

METHODS. The DNA-binding domain of GR (amino-acid residues 420–506) was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST) using pGEX-3X plasmid¹⁰. The exact amino-acid sequence of the DNA-binding domain used here is shown in Fig. 1. The DNA-binding domain was generated by polymerase chain reaction (PCR) using linearized OB10 plasmid containing cDNA encoding the β -form of human glucocorticoid¹¹ as a template. The following oligodeoxynucleotides, each with a 5' flanking *Bam*HI restriction site, were synthesized (DNA Synthesis Facilities, Univ. Alberta), and used as primers: primer A-5'-ATGGATC CTC TGC CTG GTG TGC TCT GAT GAA-3' (5'; forward primer); primer B-5'-ATGGATCC TCC TGT AGT GGC CTG CTG AAT TCC-3' (3'; reverse primer) for the synthesis of cDNA encoding the protein. Primer A corresponds to nucleotides 1,389–1,403 (underlined) and primer B to nucleotides 1,626–1,650 (underlined)¹¹. PCR was carried out for 25 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. Amplification was completed by a final incubation at 72 °C for 10 min. PCR products were digested with *Bam*HI and *Eco*RI, purified by PAGE and ligated into *Bam*HI/*Eco*RI-cut pGEX-3X to generate pGEX-GR encoding the GR DNA-binding domain. *E. coli* transformed with pGEX-GR were grown to mid-log phase and then induced to express the fusion protein with 0.1 mM isopropylthiogalactoside (IPTG). GST-GR was purified by step chromatography, first on a glutathione-Sepharose-4B affinity column¹⁰ and then by a calreticulin-affinity column. The calreticulin affinity column was synthesized by coupling purified, recombinant calreticulin¹⁰ to CNBr-activated Sepharose (Pharmacia) as recommended by the manufacturer. Chromatography was carried out using the FLPC system (Pharmacia). SDS-PAGE (10% acrylamide) was done as described¹².

amino-acid sequence of the DNA-binding domain of GR (Fig. 1a) in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein, GST-GR, and assayed for binding to calreticulin. Figure 2 shows the chromatographic profiles of GST and GST-GR fusion protein on a column of immobilized calreticulin. GST did not bind to the calreticulin-affinity column (Fig. 2a), but

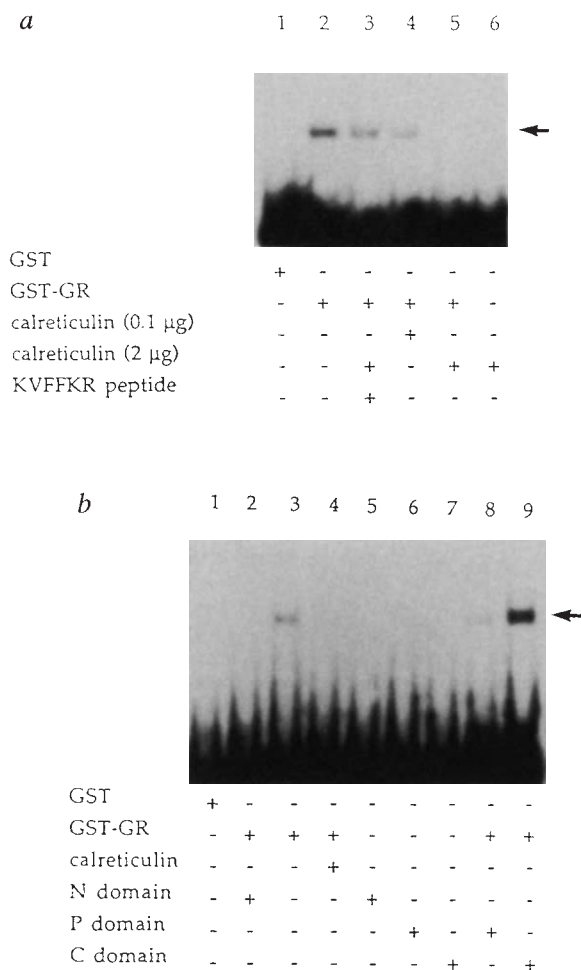


FIG. 3 Interactions of the glucocorticoid receptor DNA-binding domain with the GRE is abolished in the presence of calreticulin (a) or N domain (b). **a**, Lanes: 1, GST (2 µg); 2, GST-GR (2 µg); 3, GST-GR in the presence of calreticulin and the KVFFKR synthetic peptide (100 µg); 4, GST-GR in the presence of 0.1 µg calreticulin; 5, GST-GR in the presence of 2 µg calreticulin; 6, calreticulin alone (5 µg). **b**, The N, P and C domains were assayed for ability to inhibit GST-GR from interacting with the GRE. Lanes: 1, GST (2 µg); 2, GST-GR in the presence of 4 µg GST-N-domain fusion protein; 3, GST-GR (2.5 µg); 4, GST-GR with 5 µg calreticulin; 5, GST-N-domain (4 µg); 6, GST-P-domain (3 µg); 7, GST-C-domain (2 µg); 8, GST-GR with GST-P-domain fusion protein (3 µg); 9, GST-GR with GST-C-domain fusion protein (2 µg). Arrow indicates complex DNA.

METHODS. DNA mobility shift assay was done in 20 mM Tris, pH 7.5, 1 mM EDTA, 20 mM NaCl, 0.05% bovine serum albumin, 4 mM dithiothreitol (DTT), 10% glycerol and 2 µg poly(dI-dC)¹³. The proteins and buffer were added together and incubated at 25 °C for 15 min. Radioactive oligodeoxynucleotides were added, incubated for a further 10 min and loaded onto a 5% polyacrylamide gel in 22 mM Tris, pH 7.5, 22 mM boric acid and 0.5 mM EDTA. Complementary oligodeoxynucleotides containing the GRE were synthesized (DNA Synthesis Facilities, Univ. Alberta) and used in this assay: 5'-TCC TTG TTT TAA GAA CAG TTA TCG ATT ATA AAC-3' and 5'-GTT TAT AAT CGA TAA CGT TTC TTA AAA CAA GGA-3'. Calreticulin was isolated from fresh canine pancreas by selective ammonium sulphate precipitation¹⁴. The N, P and C domains were expressed in *E. coli* as GST fusion proteins and purified as described¹⁰.

GST-GR was specifically absorbed by the column (Fig. 2a and b; fractions 8 and 9). Interaction between calreticulin and the DNA-binding domain of GR occurred at a 1:1 molar ratio as determined by sequential non-denaturing and denaturing gel electrophoresis (S. Baksh and M.M., unpublished observations).

We next tested whether calreticulin had any effect on the ability of GR to interact with its glucocorticoid response element (GRE). Figure 3a shows that the GST-GR fusion protein interacts with a synthetic GRE in a mobility shift assay (lane 2) and that neither GST nor calreticulin alone binds to this sequence (lanes 1 and 6). The interaction of GST-GR with the GRE was blocked by addition of 5 µg calreticulin (lane 5); this inhibition could be reversed by inclusion of competing KVFFKR peptide (lane 3). No significant influence of Ca²⁺ on the interaction was seen. We conclude that calreticulin binds to the DNA-binding domain of the GR and thus prevents its interaction with the GRE.

Calreticulin can be divided into distinct structural domains, referred to as N, P and C domains¹. To establish which region of calreticulin interacts with GST-GR, we expressed and purified these regions and tested for their interaction with the GST-GR DNA-binding domain in mobility shift assays. The N-domain fusion protein blocked the binding of GST-GR with DNA in a way similar to full-length calreticulin (Fig. 3b, lane 2 and 4), whereas the P domain had very little effect on binding (lane 8); surprisingly, the binding of GST-GR to the GRE was enhanced by the C domain (lane 9). The reason for this enhanced binding is not clear, but it is not due to the C domain binding to DNA because purified N, P and C domains alone did not bind to the GRE (Fig. 3b, lanes 5–7). Therefore the interaction between calreticulin and the GR appears to be mediated through the N domain.

To assess the *in vivo* significance of the GST-GR-calreticulin interaction, we transfected mouse L fibroblasts with a GRE-luciferase reporter plasmid (GRE-luc)⁸ to determine whether cotransfection with a calreticulin expression vector (pSVL-CRT) modulated GR-mediated activation of the luciferase reporter gene. In the presence of a control plasmid (pSVL), luciferase activity increased 20-fold upon addition of dexamethasone (Fig. 4a). This glucocorticoid-dependent increase in reporter gene expression was reduced ~85% when the calreticulin expression vector was included in the transfection. To show that calreticulin effects are specific to GR-mediated transcription, we co-transfected L fibroblasts with glucocorticoid-insensitive reporter genes (luciferase and growth hormone) under the control of GRE-deleted promoters of mouse mammary tumour and Rous sarcoma viruses. Figure 4b shows that co-transfection with pSVL-CRT plasmid has no effect on the activities of these promoters, indicating that calreticulin does not sequester any general factors and/or DNA-binding proteins involved in their activation. Results were identical in HeLa cells (data not shown). Calreticulin and GR therefore seem to interact specifically inside a cell and block binding of the receptor to the GRE.

The physiological significance of these findings was analysed using mouse L cells stably transfected with an expression plasmid containing calreticulin complementary DNA in the sense orientation (cells designated KAB8-CRT; expression of calreticulin increased 2.5-fold relative to control plasmid) or in the antisense orientation (BAK4-CRT cells; expression ~1.5-fold lower than control). BAK4-CRT and KAB8-CRT cells were assayed for mRNA (Fig. 4d) and protein (Fig. 4c) products of the glucocorticoid-sensitive cytochrome P450 gene: BAK4-CRT cells showed increased expression of dexamethasone-sensitive cytochrome P450 protein and messenger RNA, whereas expression was reduced in KAB8-CRT cells containing calreticulin sense cDNA. These results also show that calreticulin can modulate transcriptional activity *in vivo*.

We have shown that calreticulin interacts *in vitro* with the DNA-binding domain of the GR and that it regulates transcriptional activity of the receptor *in vivo*. As the DNA-binding

domains are highly conserved among nuclear receptors^{4,6}, calreticulin may also interact with other steroid receptors in a similar way and so affect their activities. Dedhar *et al.*⁹ have also shown that calreticulin interacts with nuclear hormone receptors and can inhibit their function. The interaction between GR and calreticulin seems to be mediated through the N domain of calreticu-

lin, indicating that this domain is functionally important. The N domain of calreticulin is highly conserved: more than 95% identity is found in this amino-acid sequence in insect, nematode, rat, rabbit, mouse and human calreticulins¹. Our results indicate that calreticulin is not only a Ca^{2+} -binding protein but that it may also regulate gene expression during development, differen-

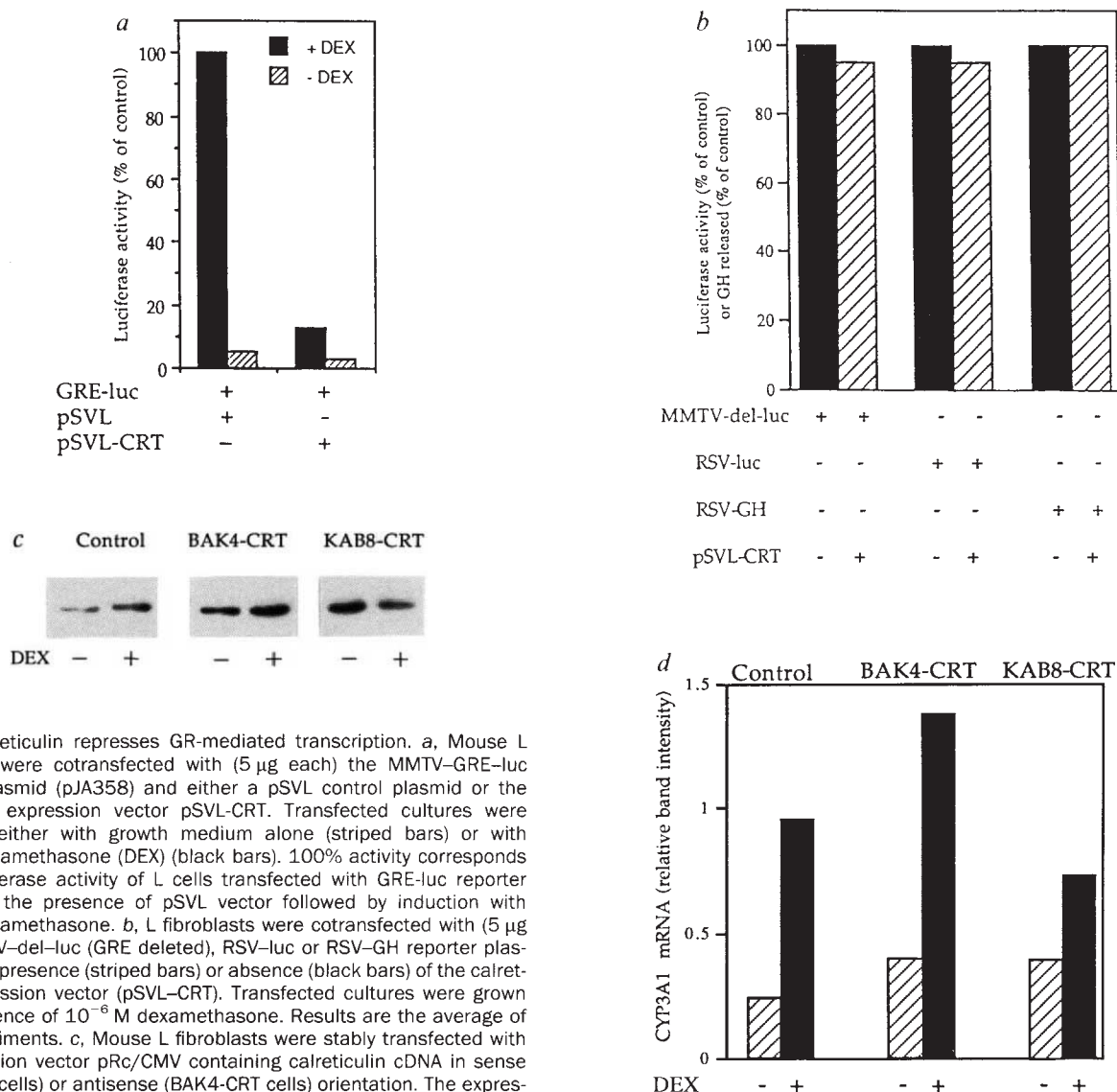


FIG. 4 Calreticulin represses GR-mediated transcription. **a**, Mouse L fibroblasts were cotransfected with (5 μg each) the MMTV-GRE-luc reporter plasmid (pJA358) and either a pSVL control plasmid or the calreticulin expression vector pSVL-CRT. Transfected cultures were incubated either with growth medium alone (striped bars) or with 10^{-6} M dexamethasone (DEX) (black bars). 100% activity corresponds to the luciferase activity of L cells transfected with GRE-luc reporter plasmid in the presence of pSVL vector followed by induction with 10^{-6} M dexamethasone. **b**, L fibroblasts were cotransfected with (5 μg each) MMTV-del-luc (GRE deleted), RSV-luc or RSV-GH reporter plasmids in the presence (striped bars) or absence (black bars) of the calreticulin expression vector (pSVL-CRT). Transfected cultures were grown in the presence of 10^{-6} M dexamethasone. Results are the average of three experiments. **c**, Mouse L fibroblasts were stably transfected with the expression vector pRc/CMV containing calreticulin cDNA in sense (KAB8-CRT cells) or antisense (BAK4-CRT cells) orientation. The expression of glucocorticoid-sensitive cytochrome P450 was determined in cells grown in the presence or absence of 10^{-6} M dexamethasone by immunoblotting with antibodies against cytochrome P450 CYP3A1. The immunological reaction was developed using Amersham's ECL detection system. **d**, Abundance of CYP3A1 mRNA was determined in control, BAK4-CRT and KAB8-CRT cells grown in the presence (striped bars) or absence (black bars) of 10^{-6} M dexamethasone. CYP3A1 mRNA levels were normalized against the corresponding signals of the ribosomal protein L32 and the glyceraldehyde 3-phosphate dehydrogenase mRNAs¹⁵.

METHODS. Mouse L fibroblasts grown in DMEM supplemented with 10% calf serum were transfected by the calcium phosphate method⁸. After a 2-min glycerol shock, cells were incubated in DMEM containing 10% charcoal-treated calf serum for 12 h, followed by incubation for 24 h either in DMEM alone or in DMEM with 10^{-6} M dexamethasone. Cells were collected and luciferase activity was determined¹⁵. L cells were also cotransfected with plasmids encoding glucocorticoid-insensitive reporter constructs including a GRE-deleted MMTV-luciferase (MMTV-del-luc), RSV-luciferase (RSV-luc) or RSV-human growth hormone (RSV-GH), incubated in DMEM containing 10^{-6} M dexamethasone spun and assayed for luciferase activity¹⁶ or growth hormone released into

the medium¹³. For stable transfections, mouse L fibroblasts were electroporated (1,500 V cm^{-1} ; 25 μF) with 20 μg pRc/CMV-CRT containing calreticulin cDNA either in sense (KAB8-CRT cells) or antisense (BAK4-CRT) orientation or with a control pRc/CMV plasmid. Cells were selected for resistance to the antibiotic G418 (400 $\mu\text{g ml}^{-1}$). Before stimulation with dexamethasone, these cell lines were grown in DMEM containing 10% charcoal-treated calf serum for 24 h, incubated for 24 h in DMEM alone or in DMEM with 10^{-6} M dexamethasone. Expression of mouse cytochrome P450 was tested in these cells by immunoblotting in the presence of 0.05% Tween-20 using a 1:1,000 dilution of the CYP3A1 antibody^{2,17}. The peroxidase reaction was developed using Amersham's ECL detection system. Northern blotting was done according to ref. 15 using as a probe the cDNA encoding cytochrome P450 CYP3A1 (ref. 18). The relative abundance of each mRNA was determined using Fujiex BAS1000 Phosphorimager. The calreticulin expression vectors (pSVL-CRT and pRc/CMV-CRT) were constructed by insertion of the cDNA encoding rabbit skeletal muscle calreticulin¹⁹ into the pSVL vector (Pharmacia) or pRc/CMV vector (Invitrogen). The pJA358 plasmid containing MMTV-GRE-luciferase reporter gene has been described⁸.

tiation and homeostasis in a wide variety of organisms ranging from *Drosophila* to humans. □

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Inhibition of nuclear hormone receptor activity by calreticulin

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WE have shown that a polypeptide of *M*, 60,000 (60K) that shares N-terminal homology with a calcium-binding protein, calreticulin, can bind to an amino-acid sequence motif, KXGFFKR, found in the cytoplasmic domains of all integrin α -subunits¹. The homologous amino-acid sequence, KXFFKR (where X is either G, A or V), is also present in the DNA-binding domain of all known members of the steroid hormone receptor family²; amino acids in this sequence make direct contact with nucleotides in their DNA-responsive elements and are crucial for DNA binding³. Here we show that both the 60K protein (p60), purified on a KLGFFKR–Sephacryl affinity matrix, and recombinant calreticulin can inhibit the binding of androgen receptor to its hormone-responsive DNA element in a KXFFKR-sequence-specific manner. Calreticulin can also inhibit androgen receptor and retinoic acid receptor transcriptional activities *in vivo*, as well as retinoic acid-induced neuronal differentia-

tion. Our results indicate that calreticulin can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors.

The conservation of the KXGFFKR sequence in the α -subunits of integrins is shown in Table 1. A computer search of the Swiss protein data bank for the presence of this sequence motif in other proteins revealed that a highly homologous sequence is present in the DNA-binding domain of all members of the nuclear hormone receptors (Table 1)^{2,4}. Because amino acids in this motif are essential for the binding of nuclear hormone receptors to their DNA-responsive elements^{3,5}, we investigated whether the 60K protein isolated by affinity chromatography on a KLGFFKR–Sephacryl affinity matrix¹ could modulate DNA binding and transcriptional activities of nuclear hormone receptors.

Although calreticulin contains a KDEL motif at its C terminus and is therefore thought to be resident in the endoplasmic reticulum^{6–8}, it also has a nuclear targeting signal^{6,8,9}, so could also be present in the nucleus⁸. The presence of p60 in nuclei was demonstrated by affinity chromatography of human osteosarcoma cell (HOS) nuclear extracts of a KLGFFKR-affinity column as described previously¹. As shown in Fig. 1, a 60K polypeptide that is immunoreactive with antibodies against calreticulin is highly enriched in total cellular extracts as well as in nuclear extracts. Indirect immunofluorescence of HOS cells or purified nuclei with anti-calreticulin antibody also demonstrated intranuclear calreticulin expression (data not shown). These results confirm the presence of a calreticulin-related p60 protein in nuclei.

To determine whether p60 can directly modulate the binding of nuclear hormone receptors to DNA through the KXFFKR sequence, the interaction of the DNA-binding domain of recombinant androgen receptor with its hormone-responsive element was analysed by gel mobility shift assay. As shown in Fig. 2, the migration of a ³²P-labelled 26-base-pair (bp) DNA androgen-responsive element at positions –115 to –140 of the rat probasin gene promoter¹⁰ was retarded by the androgen receptor DNA-binding domain, indicating the formation of a complex between

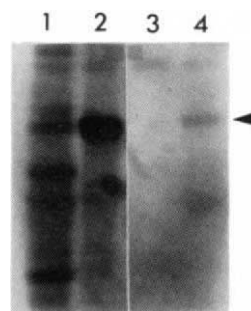


FIG. 1 Isolation of p60 from nuclei by affinity chromatography on KLGFFKR–Sephacryl. Cell extracts were prepared from whole cells or from purified nuclei and applied to KLGFFKR–Sephacryl affinity matrix. Bound proteins were eluted with EDTA and analysed by SDS–polyacrylamide gel electrophoresis¹. Separated proteins were electrophoretically transferred to nitrocellulose filters and probed with an anti-calreticulin antibody. Lanes: 1, total cellular extract; 2, EDTA-eluted material from affinity column to which total cellular extract was applied; 3, nuclear extract; 4, EDTA-eluted material from affinity column to which nuclear extract was applied. Arrowhead indicates the position of p60.

METHODS. Nuclei were purified from HOS cells as described⁹. Purified nuclei were either lysed in PBS containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and 1 mM PMSF, or were applied to a glass coverslip and stained for nuclear antigen with anti-nuclear monoclonal antibody MAB1218 (Chemicon Int., CA). Nuclei were visualized by indirect immunofluorescence. The total cellular or nuclear extracts were subjected to affinity chromatography on a KLGFFKR–affinity matrix, and p60 isolated as described¹.

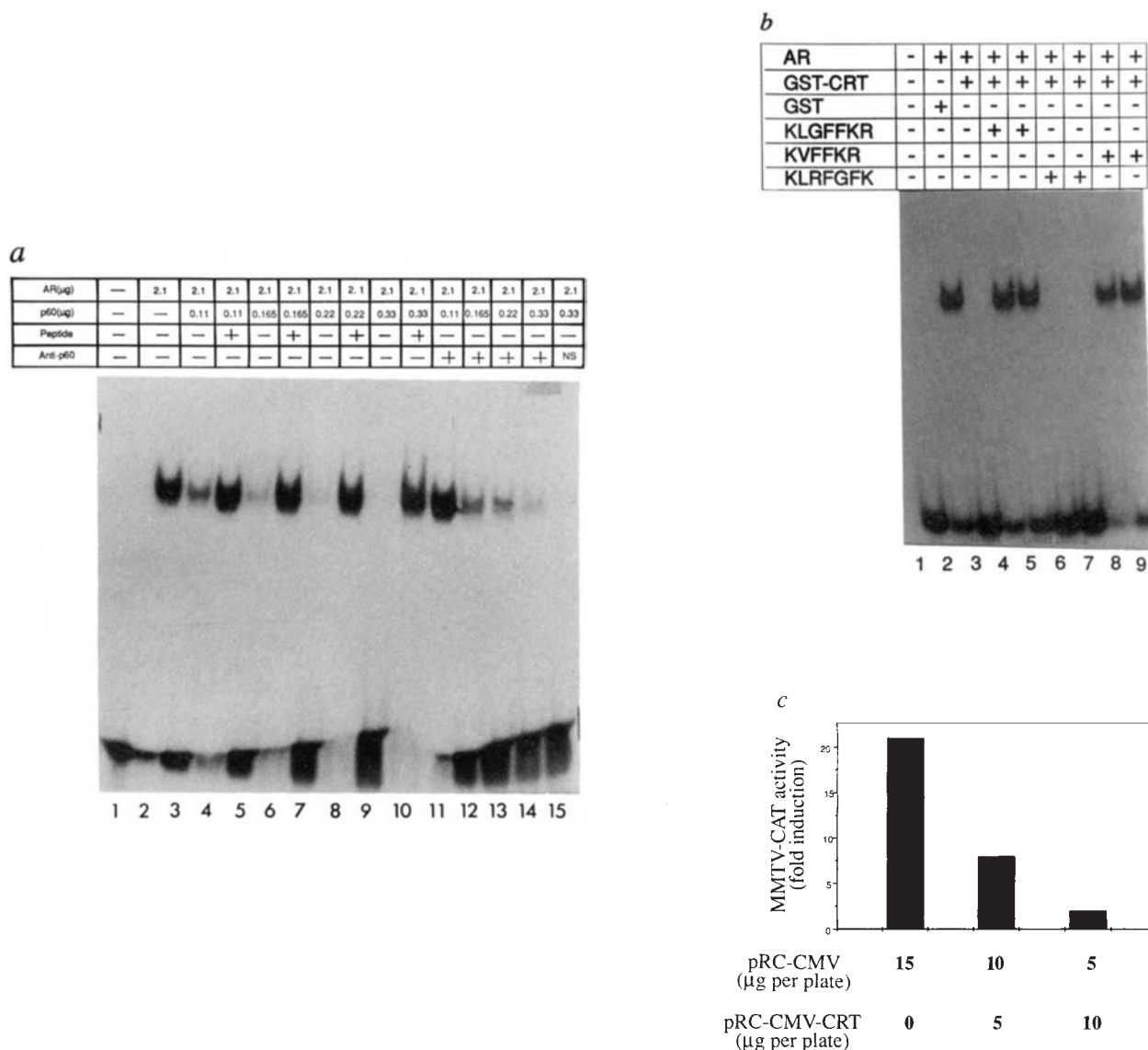


FIG. 2 Interactions between the DNA-binding domain of androgen receptor, calreticulin and DNA. Affinity-purified DNA-binding domain of the recombinant rat androgen receptor (AR) was preincubated with or without the indicated concentrations of purified p60 at 4 °C for 30 min. Reaction mixtures were then incubated with 32 P-labelled 26-base-pair androgen response element (ARE)¹⁰, and analysed by gel retardation assay¹⁰. The sequence of the ARE used was:

5' GTAAAGTACTCCAAGAACCTATTgt 3'
3' CATTTCATGAGGTTCTTGATAAca 5'

a, Lanes: 1, 32 P-labelled ARE by itself; 2, retardation of ARE by AR; 3, 5, 7 and 9, effect of increasing concentrations of p60 (0.11 μ g–0.33 μ g) on AR–ARE binding; 4, 6, 8 and 10, reversal of p60 inhibition of AR–ARE binding by 25-molar excess KLGFKKR peptide; 11, effect of addition of anti-calreticulin antibody to p60 inhibition of AR–ARE binding; 12–15, increasing amounts of p60 in the presence of anti-calreticulin antibody. Increasing the p60 concentration overcomes the effect of antibody on p60 inhibition of AR–ARE. **b**, Lanes: 1, 32 P-labelled ARE alone; 2, retardation of ARE by AR in the presence of glutathione-S-transferase (GST); 3, inhibition by GST–calreticulin (GST–CRT) of AR–ARE interaction; 4 and 5, reversal of this inhibition by KLGFKKR peptide; 6 and 7, inability of the scrambled peptide (KLRFGFK) to reverse the effect of CRT on AR–ARE interaction; 8 and 9, the peptide KVFFKR can also reverse inhibition by CRT of the AR–ARE interaction. The amount of calreticulin was 2 μ g and the peptides were in 50-fold molar excess. **c**, Inhibition of androgen-induced CAT activity by calreticulin. Vero fibroblasts were cotransfected with an MMTV-CAT reporter vector and various amounts of a calreticulin expression vector and the pRC-CMV vector alone (Inv-

trogen) using the calcium phosphate method²¹. In all transfections, 10 μ g β -galactosidase expression vector and 10 μ g of an androgen-receptor expression vector²² were included. Transfected cells were incubated in medium alone or in the presence of 100 nM R1881 (synthetic androgen) for 12 h. Cells were then lysed and CAT activity measured²². An aliquot of the cell extracts was also assayed for β -galactosidase activity. This activity was used to standardize the measurement of CAT levels in each experiment by taking into account the efficiency of the transfection. Every sample was tested in quadruplicate and the average activity calculated. CAT activity induction is defined as the ratio between the standardized CAT activity of R1881 treated cells and the corresponding untreated cultures. Vero cells were grown in α -minimum essential medium containing 10% charcoal-treated calf serum.

METHODS. As described elsewhere¹⁰, DNA-binding domain of recombinant rat AR was prepared as a GST fusion protein using the pGEX-3X vector and purified by glutathione–agarose affinity chromatography. p60 was purified by affinity chromatography on KLGFKKR–Sepharose, followed by gel electrophoresis as described¹. AR and p60 were >90 and 95% pure, respectively, as determined by SDS–PAGE and Coomassie blue staining. Recombinant calreticulin (GST fusion protein) was prepared as described¹¹. To analyse the effect of p60, or recombinant calreticulin, on receptor–DNA binding activity, AR was preincubated with p60 for 30 min at 4 °C. To analyse the effects of synthetic peptides, and of anti-calreticulin antibody and non-immune IgG on p60 inhibition of AR–ARE binding, the peptide, antibody or IgG were preincubated with p60 for 30 min at 4 °C. AR preparation was then added to these mixtures and incubated for a further 30 min at 4 °C.

the receptor and the DNA¹⁰. Preincubation of purified p60 with the recombinant receptor resulted in a dose-dependent inhibition in the formation of this complex (Fig. 2a, lanes 3, 5, 7 and 9). The sequence specificity of this inhibition was demonstrated by the finding that the inhibition by p60 of receptor–DNA binding was reversed by addition of competing KLGFFKR peptide (Fig. 2a, lanes 4, 6, 8 and 10) or KVFFKR (Fig. 2b, lanes 8 and 9), whereas a scrambled peptide (KLRFQFK) was much less

effective (Fig. 2b, lanes 6 and 7). An antibody to calreticulin, which crossreacts with p60 (ref. 1), also reversed this inhibition by p60 (Fig. 2a, lane 11), demonstrating p60 specificity. Non-immune IgG did not have any effect on the inhibition of receptor–DNA interaction by p60 (Fig. 2a, lane 15). Furthermore, the KLGFFKR peptide, anti-calreticulin antibody or non-immune IgG did not have any effect by themselves on the receptor–DNA interaction (data not shown). p60 did not affect the

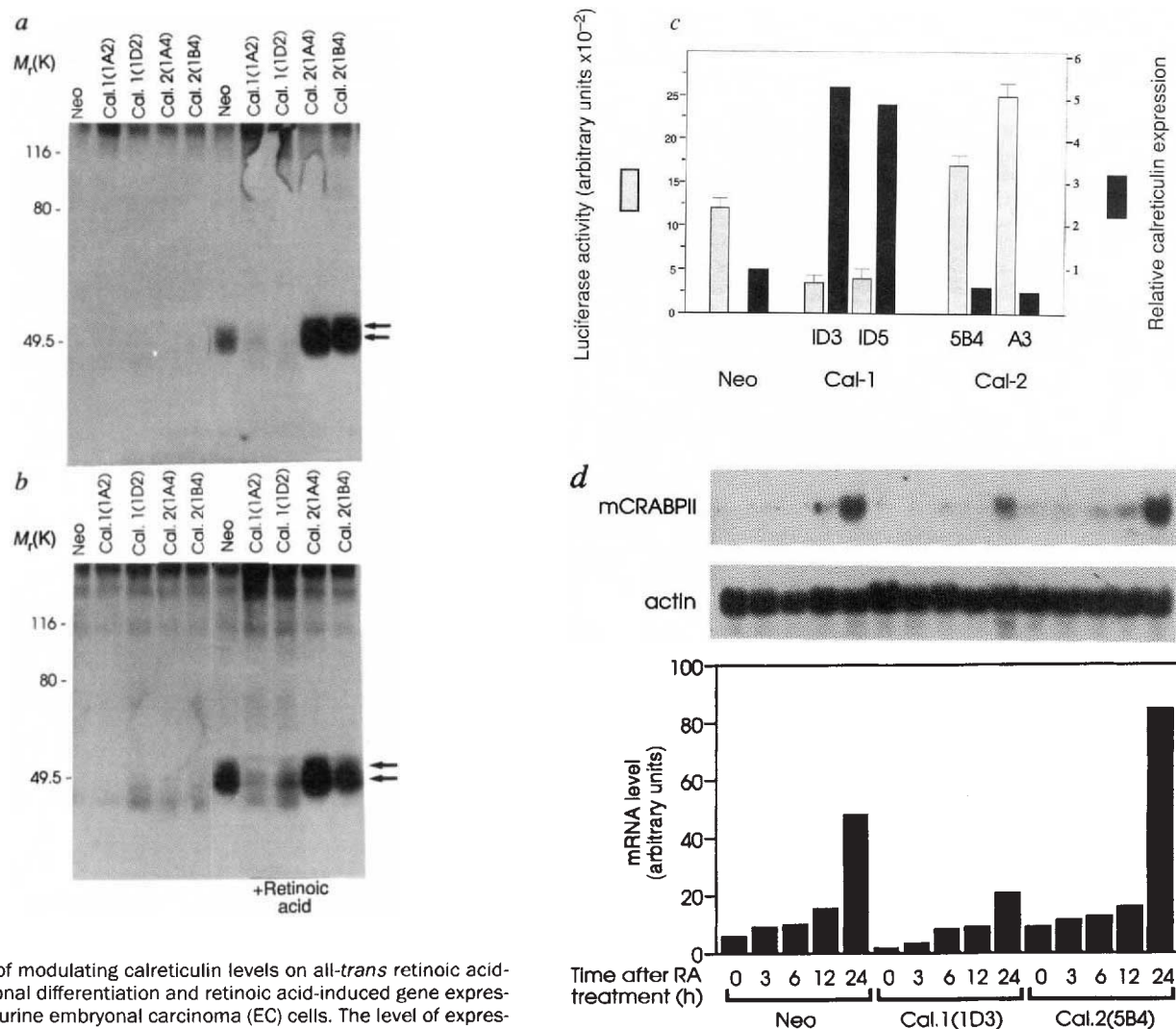


FIG. 3 Effect of modulating calreticulin levels on all-*trans* retinoic acid-induced neuronal differentiation and retinoic acid-induced gene expression in P19 murine embryonal carcinoma (EC) cells. The level of expression of calreticulin was modulated in P19 EC cells by transfection with calreticulin cDNA inserted in the sense or antisense orientation in the pRC/CMV expression vector. P19 EC cell subclones overexpressing calreticulin (Cal-1), or antisense transfectants with reduced calreticulin expression (Cal-2), as well as control transfectants (Neo), had neuronal differentiation induced by retinoic acid as described^{15,23}. The expression of neuron-specific class III β -tubulin^{16,17} was then analysed 48 h (a) or 72 h (b) after the addition of all-*trans* retinoic acid (5 μ M). Cal-1 (1A2 and 1D2) clones were transfected with pRC/CMV-containing calreticulin cDNA in the sense orientation. Cal-2 (1A4 and 1B4) clones were transfected with pRC/CMV containing calreticulin cDNA in the antisense orientation. c, Effect of levels of calreticulin expression on retinoic acid induced RARE-driven luciferase activity. d, Northern blot analysis of mouse CRABP II mRNA after retinoic acid treatment of p19 (Neo), Cal-1 and Cal-2 cells.

METHODS. Full-length 1.9-kb calreticulin cDNA²⁴ (from R. D. Sontheimer) was subcloned into pRC/CMV (Invitrogen) expression vector in the sense and antisense orientation. pRC/CMV, pRC/CMV-Cal-1 (sense), or pRC/CMV-Cal-2 (antisense) expression plasmids were then transfected into P19 embryonal carcinoma cells by electroporation. Neomycin-resistant transfectant cells were then selected by growth in the presence of 600 μ g ml⁻¹ G418 and the resistant cells were main-

tained in 100 μ g ml⁻¹ G-418. Cal-1 and Cal-2 transfectants were subcloned by limiting dilution, and the subclones screened for calreticulin expression by western blot analysis of cell lysates with an anti-calreticulin antibody¹. Retinoic acid neuronal differentiation was induced as described^{15,23} and class III β -tubulin expression was analysed by western blotting with a class III β -tubulin monoclonal antibody (TuJ1; gift from A. Frankfurter). β -RARE–luciferase transient transfections in p19 (Neo), Cal-1 and Cal-2 cells were done as described²⁵. The vector β -RARE(3) tk-LUC was constructed by linking 3 copies of the 32-bp sequence that defines the RARE upstream from the RAR- β gene^{19,20} to the minimal thymidine kinase promoter and the firefly luciferase gene. Calreticulin expression was estimated by western blot analysis of cellular lysates¹ followed by densitometric scanning. For northern blot analysis, total cellular RNA (15 μ g) from the indicated cell lines was hybridized to ³²P-labelled CRABP(II) cDNA¹⁸ at 65 °C using Rapid Hyb buffer (Amersham). The blot was stripped and reprobed with a mouse actin cDNA probe to check for equal loading of RNA. Values for relative mRNA levels were derived from quantification of the signal in each lane using a Molecular Dynamics Phosphorimager. CRABP II mRNA levels were normalized against the corresponding actin mRNA signal.

TABLE 1 Conservation of an amino-acid sequence motif in the integrin α -subunit cytoplasmic domains and in the steroid hormone receptor family

Integrins*		Steroid nuclear receptors	
$\alpha 1$	KIGFFKR	RAR- α	ACEGCKGFFRRSIQK
$\alpha 2$	KLGGFFKR	T ₃ R- β	TCEGCKGFFRRTIQK
$\alpha 3$	KCGFFKR	VDR	TCEGCKGFFRRSMKR
$\alpha 4$	KAGFFKR	GR	TCGCKVFFKRAVEG
$\alpha 5$	KLGGFFKR	MR	TCGCKVFFKRAVEG
$\alpha 6$ (A)	KCGFFKR	AR	TCGCKVFFKRAAAG
$\alpha 6$ (B)	KCGFFKR	PR	TCGCKVFFKRAMEG
$\alpha 7$	KLGGFFKR	ER	SCEGCKAFFKRISQK
$\alpha 8$ (chick)	KCGFFDR	RXR	SCEGCKGFFKRTVRK
αv	RMGFFKR	Steroid receptor TR2	TCEGCTGFFKRSIRK
Mac-1	KLGGFFKR	NGF-induced protein 1B	TCEGCKGFFKRTVQK
p150	KVGFFKR	Early-response protein NAK1	TCEGCKGFFKRTVQK
PS2 (<i>Drosophila</i>)	KCGFFNR	Chorion factor I	SCEGCKGFFKRTVRK

Abbreviations: GR, glucocorticoid receptor; MR, mineralocorticoid receptor; AR, androgen receptor; PR, progesterone receptor; ER, oestrogen receptor; RAR, retinoic acid receptor; T₃R- β , thyroid hormone receptor; VDR, vitamin D receptor; NGF, nerve growth factor.

* Sequences from ref. 1.

binding of AP-1 to DNA, and other proteins of similar size (such as bovine serum albumin) did not have any effect on the nuclear receptor-DNA interaction either (data not shown). Recombinant calreticulin (from M. Michalak)¹¹, in the form of a glutathione *S*-transferase (GST) fusion protein, also inhibited the binding of the androgen-receptor to its response element (Fig. 2*b*, lane 2); this inhibition was also reversed by KVFFKR peptide (Fig. 2*b*, lane 2), but not by a scrambled peptide KLRFGFK (Fig. 2*b*, lane 1), confirming that p60 purified on the KLGGFFKR affinity matrix and calreticulin are functionally similar in terms of binding to nuclear hormone receptors, and that a synthetic peptide, KVFFKR, can competitively inhibit the binding of calreticulin to the KVFFKR sequence of the androgen receptor.

To determine whether calreticulin also inhibited transcriptional activity of the androgen receptor *in vivo*, expression vectors containing full-length calreticulin⁶ and androgen receptor¹⁰ complementary DNAs were co-transfected into Vero fibroblasts together with a chloramphenicol acetyltransferase (CAT) reporter plasmid driven by the mouse mammary tumour virus (MMTV) long terminal repeat (LTR). MMTV-LTR contains androgen-response elements¹⁰. As shown in Fig. 2*c*, co-transfection of the calreticulin-containing plasmid resulted in a dose-dependent inhibition of CAT activity induced by the androgen receptor. Furthermore, immunoprecipitation of calreticulin from [³⁵S]methionine/cysteine-labelled androgen-receptor-transfected Vero cells resulted in the coprecipitation of the 110K androgen

receptor, indicating a direct interaction between calreticulin and the androgen receptor (S.D. and C.-Y.L.H., unpublished observations). These data show that not only can calreticulin bind to the androgen receptor DNA-binding domain and inhibit its interaction with the androgen response elements *in vitro*, but it can also inhibit the transcriptional activity of the androgen receptor *in vivo*. Although other 59K proteins have been found in complexes with several steroid hormone receptors^{12,13}, they are distinct from calreticulin, and none of them effects binding of the receptors to their DNA-responsive elements.

As shown in Table 1, the sequence KXFFKR is present in all known members of the nuclear receptor family. The region containing this sequence in the DNA-binding domains of these receptors is crucial in DNA-sequence recognition³. Thus calreticulin, by binding to this common sequence, may modulate the binding of all members of the receptor family to DNA. Indeed, calreticulin inhibits the interaction of glucocorticoid receptor with its DNA-responsive element¹⁴, and we also have found that the interaction of the retinoic acid receptor heterodimer complex (RAR/RXR) with its DNA response element is inhibited (V.G., M.S. and S.D., manuscript in preparation). To explore the physiological significance of the finding that calreticulin can bind to the DNA-binding domain of nuclear hormone receptors and modulate their transcriptional activity, we used a retinoic-acid-responsive system, namely the induction of neuronal differentiation by retinoic acid in P19 embryonal carcinoma cells¹⁵. We predicted that increased expression of calreticulin should suppress retinoic-acid-induced neuronal differentiation, whereas decreased expression may result in the release of calreticulin inhibition and allow for a more rapid rate of neuronal differentiation. As shown in Fig. 3*a* and *b*, overexpression of calreticulin (Cal-1) by calreticulin cDNA transfection in P19 EC cells dramatically suppressed neuronal differentiation, as judged by the expression of a specific early marker of neuronal differentiation, class III β -tubulin^{16,17}. In contrast, decreased expression of calreticulin (Cal-2) by antisense calreticulin cDNA transfection gave markedly enhanced expression of class III β -tubulin. The effect of calreticulin levels on retinoic-acid-induced neuronal differentiation occurs by direct regulation of retinoic-acid-responsive genes, as shown in Fig. 3*c*, which demonstrates an inverse relationship between calreticulin expression and RARE-driven luciferase gene expression. Furthermore, the endogenous regulation of expression of the retinoic-acid-responsive genes CRABP1¹⁸ (Fig. 3*d*) and RAR- β ^{19,20} (data not shown) is substantially decreased in Cal-1 transfectants, but is either unchanged or slightly increased in the calreticulin antisense Cal-2 transfectants.

Collectively, our results show that calreticulin, by binding to the conserved KXFFKR sequence in the DNA-binding domain of nuclear hormone receptors, can modulate gene expression and cellular phenotypes, for example in cell differentiation. Calreticulin may also behave as a signal modifier by translocating between the nucleus and the cytoplasm, where it binds through an identical sequence motif to the intracellular domains of the α -subunits of integrin receptors¹. □

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Molecular cloning of *Drosophila* TFIID subunits

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TRANSCRIPTION initiation factor TFIID is a multisubunit complex containing a TATA-box-binding factor (TFIID τ /TBP) and associated polypeptide factors (TAFs) with sizes ranging from M_r ~20,000 to >200,000 (refs 1–7). As a result of direct promoter interactions^{8,9}, TFIID nucleates the assembly of RNA polymerase II and other initiation factors into a functional preinitiation complex¹⁰. Although the native TFIID complex mediates both basal and activator-dependent transcription in reconstituted systems, TBP itself is competent for only basal transcription. Thus, TAFs are essential cofactors for regulated transcription. The complementary DNAs encoding the p230 (M_r 230,000), p110 and p85 subunits of TFIID have recently been cloned^{11–18}. Here we report the molecular cloning and characterization of the p62, p42, p28 and p22 subunits. These participate in a network of heterogeneous protein–protein interactions within TFIID. Sequence similarities between p62/p42 and the histones H4/H3, respectively, suggest that these subunits have a functional relationship with chromatin.

Earlier analysis of an affinity-purified TFIID from *Drosophila* designated nine TBP-associated polypeptides (of M_r s 230K, 110K, 85K, 62K, 58K, 42K, 28K, 22K, 21K) as candidate subunits^{1,7}. We now use oligodeoxynucleotide probes based on partial amino-acid sequences of the 62K, 42K, 28K and 22K polypeptides to clone the corresponding cDNAs. The p62 and p42 cDNAs encoded polypeptides with calculated M_r s of 64,322 (592 residues) and 29,314 (278 residues), respectively (Fig. 1a, b). The two p28/p22 cDNAs seem to result from alternatively spliced messenger RNAs from a single gene (Fig. 1 legend) and encode closely related polypeptides, one of which contains a subset of sequences present in the other, with M_r s of 21,523 (196 residues) and 17,630 (160 residues), respectively (Fig. 1c–e). Although the calculated M_r s were less than expected for p42, p28 and p22, the *in vitro* translation products migrated at 42K, 28K and 22K, respectively (data not shown).

To verify the identity of the cDNA-encoded products, we used the corresponding antibodies to demonstrate copurification with native TFIID. In a chromatographic analysis of partially purified TFIID on heparin-5PW, the gradient elution profiles of endogenous immunoreactive 62K, 42K, 28K and 22K polypeptides matched the profiles of TBP (Fig. 2a), the previously characterized 230K, 110K and 85K subunits (data not shown), and the TFIID transcription activity (Fig. 2a). As expected, the antibody against recombinant p28 reacted with both p28 and p22 (Fig. 2 legend). Moreover, antibodies against the cDNA-encoded polypeptides also reacted with the corresponding components in an almost homogenous preparation of affinity-purified TFIID (Fig. 2b). From these results we conclude that our isolated cDNAs encode the *Drosophila* TFIID subunits.

The possibility that there may be functionally different species of TFIID has been suggested by the existence of functionally distinct TATA elements (see ref. 19, for example), the presence

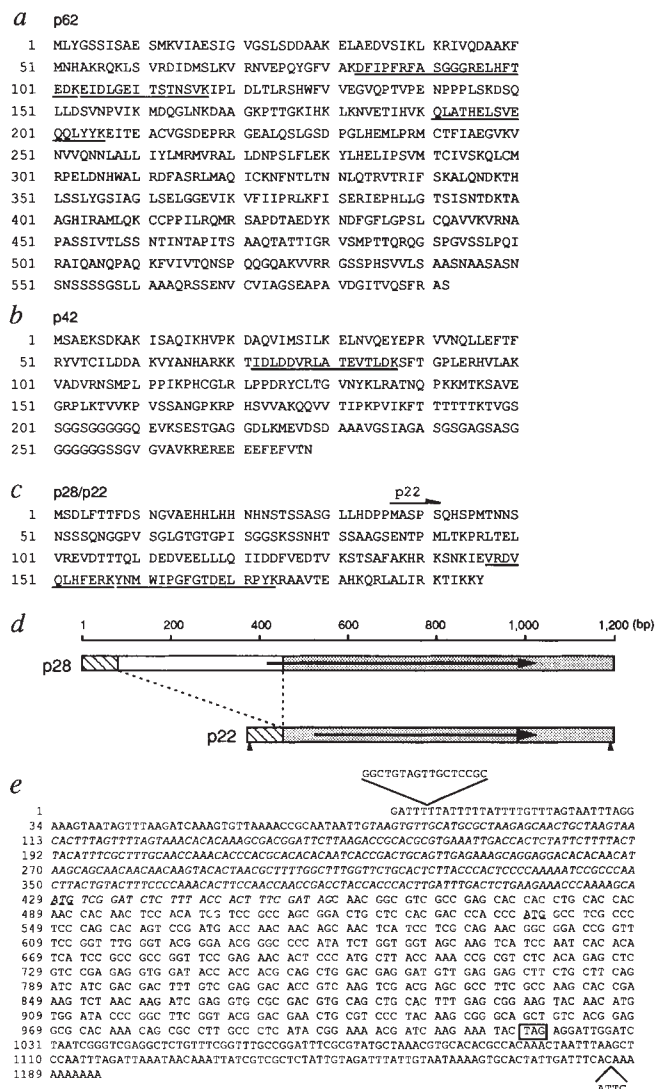


FIG. 1 Primary sequences of TFIID subunits. Encoded polypeptide sequences of a, p62; b, p42; c, p28 and p22 cDNAs. The 22K subunit is an N-terminal truncated form of the 28K subunit (see also d and e); the N terminus of p22 is indicated by an arrow in c. The positions corresponding to the experimentally determined protein sequences are underlined in a–c. d, Alignment of cDNAs encoding p28 and p22. Both cDNAs have identical sequences except that the longer clone has a 382-bp insertion (open bar), whereas the shorter one has 17- and 4-bp insertions at the 5' and 3' ends (arrowheads), respectively. The position of the open reading frame in each cDNA is indicated by an arrow. e, Nucleotide sequences of cDNAs encoding p28 and p22. The 382-bp insertion in p28 cDNA is indicated in italics; 17- and 4-bp insertions in p22 cDNA are bracketed. The 382-bp segment is bounded by consensus splice junction sequences and contains an in-frame methionine codon which could account for the larger size of p28. Together with a single band on a Southern blot of *Drosophila* genomic DNA (data not shown), these observations indicate that the two cDNAs are encoded by a single gene and are produced by alternative processing of mRNA. The predicted site for translation initiation of each protein is underlined, and the stop codon is boxed. Nucleotide sequence numbering is based on p28 cDNA.

METHODS. Polypeptides present in affinity-purified TFIID were separated by SDS-PAGE (compare Fig. 2a) and microsequenced. Using previously described methods¹¹ a *Drosophila* embryo cDNA library (provided by T. Kornberg) was screened with oligodeoxynucleotides based on derived peptide sequences to give cDNAs encoding p62, p42, p28 and p22 polypeptide sequences (a–d).

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of variant forms of TBP in plants²⁰, and studies of crude TFIID-containing fractions⁵. Our discovery of the highly related p28 and p22 subunits having variable amino termini indicates that they might be part of functionally distinct TFIID species. Such a diversity, which might also result from heterogeneity in other subunits, could provide another level of genetic control.

To investigate possible subunit-subunit interactions in TFIID, recombinant TAFs were expressed in Sf9 cells with baculovirus vectors as either histidine- or FLAG-epitope-tagged proteins. Pairwise interactions were assayed either by direct co-immunoprecipitation of co-incubated TAFs or by binding of one TAF to a second FLAG-tagged TAF conjugated to an anti-FLAG antibody attached to agarose beads. Interactions were performed under stringent binding conditions (0.5 M KCl, 0.1% NP-40) and the immunoprecipitated or conjugate-bound complexes were washed under the same conditions before elution and immunoblot analysis with antibodies against individual TAFs. Controls included the use of pre-immune antibodies for immunoprecipitation and the use of TAFs lacking FLAG epitopes in the respective analyses. First, assays with recombinant FLAG-p62 revealed strong interaction of p62 with p42 and with TBP (odd-numbered lanes in Fig. 3a and b), whereas no interaction was evident in controls using p62 without the FLAG epitope (even-numbered lanes in Fig. 3a and b). Interaction was absent or very weak for p62 with p110 (Fig. 3c), p230 or p85 (data not shown). Second, direct co-immunoprecipitation revealed a strong interaction of p42 with TBP using either anti-TBP (Fig. 3d) or anti-p42 (data not shown) antibodies. In each case, co-immunoprecipitation was dependent on the specific antibody and the presence of both p42 and TBP. In contrast, p42 showed only very weak interactions with p230, p110 and p85 under similar conditions (data not shown). Third, p28 and p22 each interacted strongly with TBP (Fig. 3e, g) and p110 (Fig. 3f, h). The specificity of these TBP and p110 associations is further demonstrated by the lack of a detectable TBP-p110 interaction in the

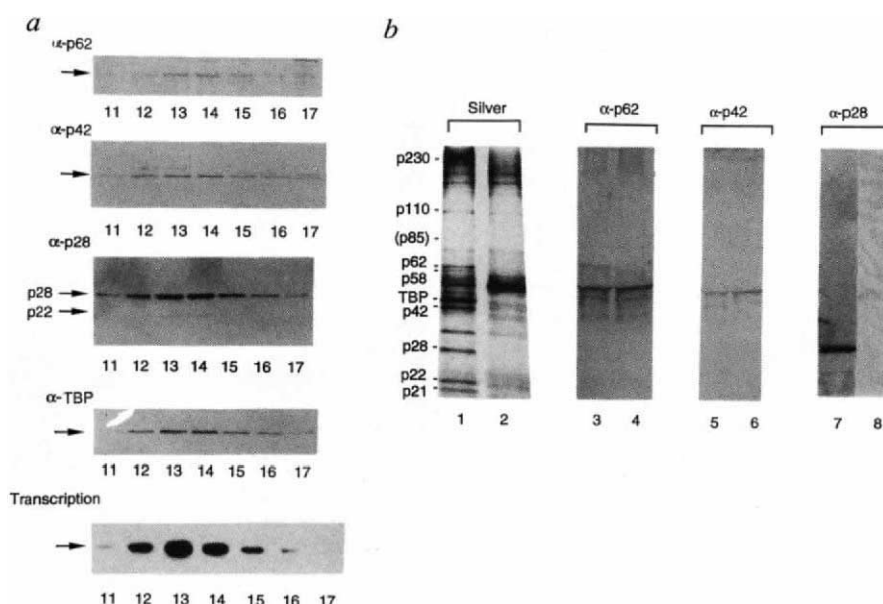
FLAG-epitope-based assay (Fig. 3i). Although previously TBP seemed to interact strongly only with p230 (ref. 7), as a result of improved methodology it is now evident that TBP can also associate with p85 (ref. 17) and with p62, p42, p28 and p22 (Fig. 3b, d, g, i).

Our results show that each of the newly cloned subunits tested, as well as TBP, can interact in pairwise combinations with several subunits in a network of interactions within TFIID (Fig. 3j). Some of these interactions may serve to hold the subunits together, whereas others may be subject to dynamic change induced by regulatory factors. In this regard, we have identified an 81-residue domain of p230 that can inhibit TATA-box binding of TBP and which is indistinguishable from the sequence that binds TBP (T.K., S. Yamashita, M.H., R.G.R. and Y.N., unpublished observation). The interaction of the p230 N-terminal region with TBP might therefore directly control promoter binding. As native TFIID can bind independently to the TATA elements of at least some promoters (for example, see refs 4, 6, 8, 9, 21, 22), our observations indicate that the inhibitory p230-TBP interaction is probably subject to regulation by other TFIID subunits and/or by transcriptional activators and repressors.

Comparison of cDNA-encoded protein sequences with protein databases (Fig. 4 legend) revealed significant similarity between p62 and histone H4 (Fig. 4a) and between p42 and histone H3 (Fig. 4b). Histones H3 and H4 are important in the formation and function of the nucleosome core. This consists of 1.75 turns of DNA (146 base pairs) wrapped round an octameric complex of two each of histones H2A, H2B, H3 and H4; the H3/H4 tetramer alone is responsible for organizing the core 120 base pairs of DNA into the arrangement found in the octameric complex²³. The globular C-terminal domains of H3 and H4 are sufficient for tetramer formation and for DNA organization²³; these both contain a long helix flanked on either side by a loop segment and a short helix²⁴. p62 and p42 can be aligned with

FIG. 2 Identification of cDNA products as TFIID subunits. **a** Polypeptides that are identical in size and immunologically related to the cDNA products cochromatograph with TBP and with TFIID activity. Heparin-5PW HPLC fractions were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with either anti-TBP antibody or anti-cDNA product antibody. Functional TFIID activity in each fraction was measured in a TFIID-dependent reconstituted transcription system⁷. The expected positions for each subunit and the specifically initiated transcription product are indicated by arrows. Numbers indicate fraction numbers. **b** Antibodies against recombinant cDNA-derived polypeptides crossreact with affinity-purified TFIID components. TFIID was affinity-purified⁷ using protein A-Sepharose with anti-TBP antibody (lanes 1, 3, 5 and 7) or with non-immune antibody (lanes 2, 4, 6 and 8). Purified proteins were separated by SDS-PAGE and visualized by silver staining (lanes 1 and 2) or by immunostaining with antibody against recombinant p62 (lanes 3 and 4), p42 (lanes 5 and 6) or p28 (lanes 7 and 8). Previously identified tightly associated polypeptides

(p230, p110, p85, p62, p58, p42, p28, p22, p21) are indicated. p85 was seen more clearly in other experiments¹⁷; p110 migrated just above the nonspecific band. In **a** and **b**, antibody raised against the recombinant 28K polypeptide reacted (as expected) with both 28K and 22K endogenous proteins. Although the signal was ~10-fold greater for p28 than for p22, this does not correspond to a greater abundance of p28 because the specific activity of the antibody (as determined with



recombinant protein standards) is 4-fold higher for p28.

METHODS. Rabbit antibodies were prepared against recombinant histidine-tagged fusion proteins expressed in *E. coli* (p28) or via baculovirus vectors in Sf9 cells (p62 and p42), and purified first by Ni-agarose affinity chromatography and then by excision from SDS-polyacrylamide gels. Fractionation of TFIID, immunoblotting and transcription have been described^{7,11}.

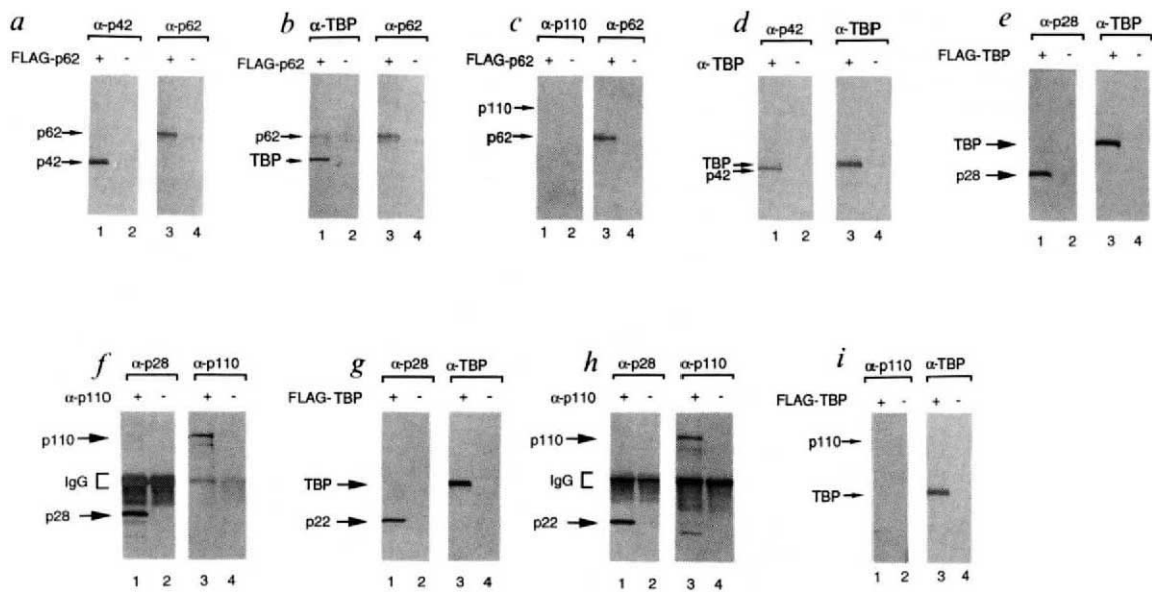
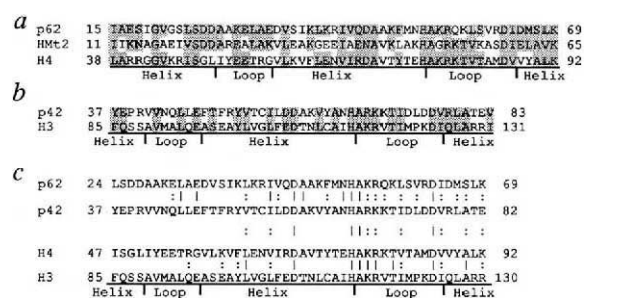


FIG. 3 Subunit interactions in the TFIID complex between: a, p62 and p42; b, p62 and TBP; c, p62 and p110 (none); d, p42 and TBP; e, p28 and TBP; f, p28 and p110; g, p22 and TBP; h, p22 and p110; i, TBP and p110 (none). For interaction studies with anti-FLAG antibodies (a, b, c, e, g and i), symbols above lanes indicate that FLAG-tagged p62 or TBP (+) or histidine-tagged p62 or TBP (–) were used. For direct co-immunoprecipitation studies (d, f, and h), symbols indicate immunoprecipitation with anti-TBP or anti-p110 antibodies (+) or corresponding preimmune sera (–). Antibodies (α -) used for immunoblotting of the corresponding interacting factors are indicated at the top. j, Potential interaction network within the TFIID complex. Overlap between subunits is based on interactions deduced from this and other studies^{11,15,17}. Subunits p230, p85, p28 and p22 can each interact directly with p110 and TBP; p62 and p42 can interact with each other and also with TBP; p230 has at least two TBP-binding sites, a C-terminal site that binds weakly and an N-terminal site that binds strongly and which suppresses the TATA-box-binding activity of TBP (T.K., S. Yamashita, M. H., R.G.R and Y.N., unpublished observation). It is not known whether p28 and p22 are members of the same or different TFIID complexes. As the interactions indicated for a given subunit (such as TBP) with several other subunits were deduced from pairwise associations, these could represent alternative arrangements in functionally distinct TFIID species or be a result of changes in TFIID structure during regulation of initiation. METHODS. Fusion proteins containing either the FLAG epitope (DYKDDDDK) or 6 histidine residues (inserted just after the initiating methionine in each case; 12th codon was used as an initiating methionine in p62) were expressed using baculovirus vectors in Sf9 cells. For

immunoaffinity analysis with anti-FLAG antibodies, whole-cell lysates were incubated with anti-FLAG M2 antibody–agarose (IBI/Kodak) at 4 °C for 1 h. Beads were washed three times with 0.5 M KCl in buffer C (25 mM HEPES, pH 7.6, 0.1 mM EDTA, pH 8.0, 12.5 mM MgCl₂, 10% glycerol, 0.1% NP-40, 1 mM DTT) and further incubated with the indicated recombinant proteins in the same buffer at 30 °C for 1 h. Protein was eluted with EGTA buffer (0.1 M glycine-HCl, pH 2.5, 50% (v/v) ethylene glycol, 10% (v/v) Tween-20) after washing three times with 0.5 M KCl in buffer C, precipitated with acetone, and analysed by SDS–PAGE. Note that beads for both the FLAG epitope and control analyses contained equivalent high levels of bound antibody protein and that the amount of recombinant FLAG protein bound in the first case was ~0.2% of the total protein. For direct co-immunoprecipitation with antibodies against recombinant polypeptides, purified recombinant histidine-tagged proteins were mixed, incubated, and then precipitated^{15,17}. Typically 5–20% of the input antigen was immunoprecipitated and ~1–10% of the second polypeptide was coprecipitated.

FIG. 4 The 62K and 42K TFIID subunits have sequence similarities with histone families. a, Sequence alignment of p62 with the histone H4 family. Similarities to the H4 family are found only in the N-terminal region of p62. Shading indicates residues identical to or conserved in the p62 sequence. Hmt2 (ref. 26) is a methanobacterium thermoautotrophic histone-like protein; sequence labelled H4 is the H4 consensus sequence²⁵. b, Sequence alignment of p42 with histone H3. Sequence similarity with H3 is found only in the N-terminal regions of p42. 'H3' represents the H3 consensus sequence²⁵. c, Pairwise alignment of histones H3/H4 and p62/p42. Identical residues are indicated by bars and conserved residues by colons. Identical or conserved residues among the four sequences are shown between the pairwise alignments. The proposed protein structures²⁴ are shown below the sequences.

METHODS. Before database searches, all protein sequences were analysed for regions of low complexity. As such regions confound the statistical basis of the search algorithms and generate artefacts, they were filtered from the protein sequences²⁹. The sequence similarities are statistically significant, as indicated by the poisson probabilities for



these matches in searches of the entire NCBI non-redundant database using the BLAST algorithm³⁰ (p62 versus Hmt2: $P=9 \times 10^{-5}$; Hmt2 versus H4: $P=1.6 \times 10^{-5}$; three-way matches (BLAST3 algorithm) between p62, H4 and Hmt2: $P=3.5 \times 10^{-7}$; between p42, H3 and Hmt2: $P=3.0 \times 10^{-2}$; between p42, H4 and Hmt2: $P=8.3 \times 10^{-2}$).

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regions corresponding to the C-terminal domains of histones H4 and H3, respectively (Fig. 4a, b). Between histones H3 and H4, 16 out of 46 residues are conserved in these regions, whereas 22 out of 46 residues are conserved between p62 and p42. Remarkably, 12 residues are conserved among the four proteins. These results suggest that TFIID subunits and histone proteins might share some common function. Conservation is extensive in the loop segment which may interact with DNA in the nucleosome²⁵, in which case the p62/p42 subunits could behave like histones H3/H4 in wrapping DNA into a nucleosome-type structure. This would be consistent with the observation (Fig. 4a) that p62 shows the greatest sequence similarity, among the H4 family, with the archaeobacterial protein HMT2 (ref. 26): HMT2, like p62, lacks an N-terminal basic tail but can still form multimeric complexes and nucleosome-like structures. It is not yet known whether p62 and p42 form dimers or tetramers in native TFIID.

A central question in eukaryotic transcriptional regulation is how the repressive chromatin structure is unfolded during transcriptional initiation. In this regard, the sequence relationships between the interacting p62 and p42 subunits of TFIID and the interacting H4 and H3 core histones, respectively, may be informative. For example, p62/p42 interactions with DNA could facilitate the displacement of nucleosomal histones H3/H4 and/or maintain a compacted DNA structure near the transcription start site. Consistent with this idea, TFIID interacts with downstream promoter regions as a nucleosome does, presenting sites hypersensitive to DNase I about every 10 base pairs^{8,9}. Moreover, prior binding²⁷ and activator-induced binding²⁸ of TFIID to the promoter both prevent chromatin-mediated repression in coupled transcription-nucleosome assembly assays, although stable binding of TFIID to a preassembled chromatin template has not yet been demonstrated *in vitro*. Further study of the (re)organization of DNA by TFIID in its chromatin environment should provide insight into the mechanism of transcriptional regulation. □

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The art of intercalation

The biological relevance of the i-motif comes under examination with the structural characterization of oligonucleotides formed of repeats of the human telomeric C-strand sequence d(CCCTAA)_n.

FOR all its apparent chemical simplicity, DNA can adopt a surprising range of structures *in vitro*. One of the icons of the twentieth century, the right-handed antiparallel double helix, can exist in a number of conformations (A- and B-forms being the best known). More exotic structures include Z-DNA, a left-handed double helix, triple helices, where a third strand nestles in the major groove of a standard double helix, and the quadruple helix of the guanine tetraplex.

Although the biological importance of some of these structures is beyond doubt, the status of others is less clear. A paper from Shawn Ahmed and colleagues in this month's *Nature Structural Biology*¹ now provides the first hint that a newly characterized four-stranded structure, the cytosine-protonated cytosine (C-C⁺) tetraplex or i-motif², may be involved in telomere formation and nucleic-acid self-recognition.

The best characterized of the four-stranded structures are the guanine quartets³. These consist of four guanine bases, in a square-planar arrangement, which cohere by Hoogsteen base-pair interactions. Stacks of these G-quartets, stabilized by monovalent cations, readily form *in vitro* under physiological conditions (figure). The complexes are polymorphic; they can be formed from one, two or four separate DNA, or RNA, strands which can be in a parallel, or antiparallel, orientation relative to one another. The connectivity of the strands can vary, as can the glycosidic conformation — *syn* or *anti* — of the guanine bases.

A variety of biologically important guanine-rich sequences are known to form G-quartet structures *in vitro*. Telomeric DNA, which caps the ends of linear eukaryotic chromosomes, consists of simple tandem repeats that are G-rich on one strand, which protrudes as a single strand by two-repeat-units at the

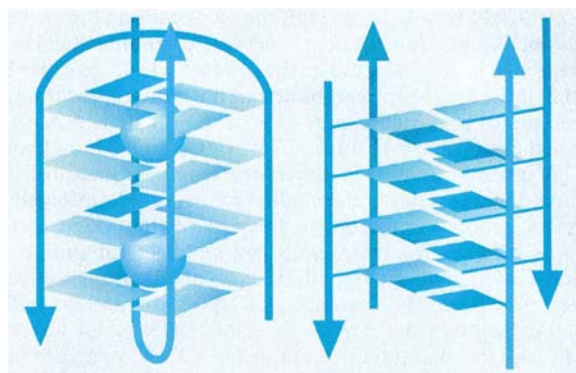
very tip of the chromosome⁴. Indeed, the structures of a number of the telomeric sequences that form G-tetraplexes have been determined by X-ray crystallography and nuclear magnetic resonance spectroscopy (ref. 5 and references therein). Do these structures exist *in vivo*? Evidence from the study of telomere-binding proteins^{6,7} and the dimerization of retroviral RNA genomes⁸ indicates that they probably do.

For every stretch of G-rich sequence capable of forming G-quartet structures in a DNA double helix, there is a complementary strand rich in cytosine bases. What is the fate of these sequences? Last year's report² of an unusual structure, the i-motif, formed in solution by d(TCCCCC)₄ suggested that such tracts of DNA rich in cytosine may in fact have a highly unusual structure.

The i-motif, like the G-quartet, has a backbone of four strands, which may all be part of the same DNA molecule or formed as dimers or tetramers. Here the similarity ends. Hemi-protonated cytosine bases participate in non-Watson-Crick base-pairs, forming parallel-stranded C-C⁺ duplexes. In the i-motif structure two such duplexes intertwine in an antiparallel fashion, with the C-C⁺ base pairs of one duplex fully intercalated with those of the other duplex (figure).

Does this addition to the pantheon of nucleic-acid structures have any biological ramifications? Clearly, the first place to look would be on the C-rich strand of telomeric DNA. Ahmed *et al.*¹ have done just this and show, using native gel electrophoresis, that the human telomeric C-strand oligonucleotide d(CCCTAA)₂ forms a dimer and d(CCCTAA)₄ forms an intramolecular complex at low pH consistent with the formation of four-stranded structures. More notably, NMR spectroscopic analysis of the d(CCC-TAA)₂ dimer reveals imino proton spectra characteristic of C⁺ imino protons, and connectivities considered to be diagnostic of DNA molecules that form the i-motif — it is, therefore, a C-C⁺ tetraplex.

The demonstration that human telomeric C-rich sequences adopt an i-motif con-



Diagrams of a guanine tetraplex (left), the spheres representing monovalent cations, and a C-C⁺ tetraplex (right). The DNA backbone is shown as solid lines with arrowheads which indicates the 5'→3' polarity of the strand.

formation suggests that this structure may be of relevance *in vivo*. G-tetraplex and i-motif structures could act in concert; any sequence, such as telomeric DNA, that will form a G-tetraplex on one strand potentially has the ability to form a C-C⁺ quadruplex on the other strand. Indeed, one structure might promote the formation of the other. G-tetraplexes have been suggested to mediate self recognition, telomere formation, meiotic chromosome pairing and recombination; the i-motif has the potential to do the same.

An obvious drawback to such speculation is the low pH, and cytosine protonation, needed for formation of the i-motif. Of course, it is possible to invoke i-motif-binding proteins, super-helical stress and so on as conditions that might yield the structure. Whether such conditions exist *in vivo* will only become evident after further experiment. Perhaps of equal interest are details of the chemistry of the i-motif (how the tetraplex folds to its final form, for example), and the answers to such questions should be just as illuminating.

Guy Riddihough

Guy Riddihough is Editor of *Nature Structural Biology*.

Also in this month's *Nature Structural Biology*: two alternative conformations of a 2-aminofluorene DNA duplex; the binding site of GrpE on DnaK; the structure of a 'cysteine ring' — a disulphide bond linked by a *cis*-peptide bond; correlated intramolecular motions and diffuse X-ray scattering in lysozyme crystals; the common fold of the matrix metalloproteinases — structures of the catalytic domains of fibroblast and neutrophil collagenase and stromelysin-1.

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The Reference Library System — sharing biological material and experimental data

Günther Zehetner and Hans Lehrach

Cosmid, yeast artificial chromosome (YAC), P1 and complementary DNA (cDNA) libraries are distributed on high-density filters to the scientific community and experimental results are stored in a common object-based database accessible through the Internet.

THE Reference Library System¹ is a model for the idea of using shared biological material, which allows participating scientists to combine the results of their individual experiments in a common pool, to connect the separate information in one database and to analyse these data much more efficiently.

For this approach, it is essential that experimental results are generated using well defined experimental materials (for example, clones or probes), as each clone is unique and can therefore only be described if the same clones or clone collections are used in the different experiments. We started therefore to generate and distribute Reference Libraries, collections of clones that are permanently stored in microtitre plates, allowing the unique identification of each clone by its address in a microtitre plate. Libraries stored in this fashion can be easily replicated and used to prepare high-density filter grids²⁻⁴ carrying up to 40,000 clones per 22 × 22 cm nylon membrane. Clones can be spotted as DNAs, as polymerase chain reaction (PCR) products⁵ or as bacterial or yeast cultures, which are then converted into DNA by colony lysis. Alternatively, PCR pools can be constructed and used in the identification of specific clones by PCR techniques. In order to be able to collect all those data generated by hybridizations of various probes to these Reference Library filters and to manage the administration of the ever-growing demand for filters from outside laboratories, we have designed a relational database system, the Reference Library DataBase (RLDB), which stores all (hybridization or PCR) results and administers the distribution of filters and clones.

Distribution

The RLDB freely distributes the clones and libraries (in the form of high-density filters) that have been included into the Reference Library System to other laboratories for non-commercial use in research projects (see Table 1). Up to now, all cosmid and P1 filter grids have been made available at no cost, with the expenses covered partly by grants from the European Community (EC). Similarly, distribution of YAC library filters

within the EC has been carried out without charging users. To cover the cost of distributing YAC filters and clones to laboratories outside the EC, we have requested reimbursement of the costs of filter construction and mailing (currently UK£435, US\$600 per filter set).

In general, the use of this service does not constitute a collaboration. We do, however, retain the right to request co-authorship in the case of as yet unpublished libraries, libraries involving a larger effort (for example, YAC filters), as well as large-scale use of libraries or filters in large-scale mapping projects.

We require that the origin of Reference Library clones are acknowledged in publications and that the official RLDB clone name is used. Clones and filters can be redistributed to other laboratories as long as these rules are included and we are informed.

Confidentiality

Probes used in hybridizations to Reference Library filters can be declared confidential. In these cases, information about the probe and its hybridization results are not included in the publicly available list of probes for an initial period of 6 months. On request, this period of confidentiality can be extended to a maximum of 12 months.

Using the system

Filters of specific libraries (see Table 2) can be requested by contacting the RLDB by mail, fax or e-mail and will be dispatched as

soon as possible thereafter. After the filters are hybridized with specific probes at the recipient laboratory, the *x-y* coordinates of the positive signals in the spotted grid are determined on an autoradiograph of the filter. These *x-y* coordinates are then sent back to the RLDB together with a description of the probe used and the identification numbers of the filters used in the hybridizations.

This information is entered into the database, the microtitre plate positions of the potential positive clones are calculated and the clones are picked into stabs and returned to the researcher together with any non-confidential information about previously identified probes hybridizing to the same clone. Clones are usually sent within 3 weeks after the hybridization results have been received.

Using the original probe the clones received are re-screened in order to confirm the positive clones and to eliminate false positives. One individual colony of each confirmed positive clone should be returned to us together with the results of the re-screen, allowing us to update the information in the database, and to include the clone into a collection of characterized clones.

In order to avoid duplication of work the publicly available list of probes and clones (updated weekly) can be checked for clones previously identified by other users of the system (see Table 3). If a probe of interest is found, the correspon-

TABLE 1 Number of probes, clones and filters for each type of Reference Library

RL Libraries	Cosmid	P1	Human YAC	Mouse YAC	cDNA
No. of probes used	1,145	117	562	227	59
No. of clone requests	1,375	135	733	260	67
No. of clones identified	32,111	1,126	6,739	4,740	1,752
No. of filter requests	280	62	233	98	31
No. of filters distributed	788	290	552	278	135

The number of requests for clones and filters from the Reference Library System continued to increase significantly over the past year and up until the end of 1993 more than 2,250 filters and 22,100 clones were distributed. Over 400 scientists have used 2,150 probes in hybridizations to the gridded libraries and sent in more than 2,720 clone and 800 filter requests.

TABLE 2 Filters from the following libraries are available

Library number	Library name	Host	Description
<i>Cosmid</i>			
50	DROCRETA	<i>E. coli</i> 1046	<i>Drosophila</i> specific cosmid library
60	L4/ <i>S. pombe</i>	ED8 767	<i>S. pombe</i> specific cosmid library
61	L4/B/ <i>S. pombe</i>	ED8 767	<i>S. pombe</i> specific cosmid library
100	L4/FSC X	ED8 767	Chromosome X specific cosmid library
101	L4/FSC X/LA	ED8 767	Chromosome X specific cosmid library
102	L4/FS21	ED8 767	Chromosome 21 specific cosmid library
104	L4/FSC X	DH5 alpha MCR (BRL)	Chromosome X specific cosmid library
105	L4/FS17	DH5 alpha MCR (BRL)	Chromosome 17 specific cosmid library
106	L4/FS22	DH5 alpha MCR (BRL)	Chromosome 22 specific cosmid library
107	L4/FS11	DH5 alpha MCR (BRL)	Chromosome 11 specific cosmid library
108	L4/FS13	DH5 alpha MCR (BRL)	Chromosome 13 specific cosmid library
109	L4/FS6	DH5 alpha MCR (BRL)	Chromosome 6 specific cosmid library
111	L4/FS18	DH5 alpha MCR (BRL)	Chromosome 18 specific cosmid library
112	L4/FS1	DH5 alpha MCR (BRL)	Chromosome 1 specific cosmid library
113	L4/FS7	DH5 alpha MCR (BRL)	Chromosome 7 specific cosmid library
<i>cDNA</i>			
507	HBf cDNA	XL1 blue (<i>E. coli</i>)	Human fetal brain cDNA
510	MBR cDNA	XL1 blue (<i>E. coli</i>)	Mouse adult brain cDNA
<i>P1</i>			
700	P1 Human	NS3145	Total genomic P1 human library
<i>YAC</i>			
900	4X YAC	<i>S. cerevisiae</i> AB1380	Human YAC library
901	4Y YAC	<i>S. cerevisiae</i> AB1380	Human YAC library
902	C3H YAC	<i>S. cerevisiae</i> AB1380	Mouse YAC library
903	C57 YAC	<i>S. cerevisiae</i> AB1380	Mouse YAC library
904	CEPH YAC	<i>S. cerevisiae</i> AB1380	Human CEPH YAC library
905	HD1	<i>S. cerevisiae</i> AB1380	Huntington Disease library
907	Pig YAC	<i>S. cerevisiae</i> AB1380	Pig YAC library
909	St Mary's mouse YAC RAD52	<i>S. cerevisiae</i> AB1380	Mouse YAC library from female C57BL/10
910	Whitehead mouse YAC I	<i>S. cerevisiae</i> AB1380	Large insert mouse YAC library

YAC and P1 libraries are from total human or mouse DNA whereas the human cosmid libraries are chromosome-specific from flow-sorted chromosomes.

ding clones can be requested from the RLDB.

Access

The RLDB contains a public and a private dataset. The public data (without those data that are kept confidential for a limited time) are made available to the scientific community in several ways. Lists of all used probes, their origin, chromosome location and number of identified potential or confirmed cosmid or YAC clones are generated once a week and automatically downloaded to the National Center for Biotechnology Information (Bethesda, Maryland) data repository. An IRX (Information Retrieval Experimental Workbench) database is created every week from the RLDB and also made available from the data repository by anonymous ftp. Table 3 gives the addresses of all different locations from where RLDB data are available.

RLDB2

In order to store data from a wider range of experiments, we have designed a new database (RLDB2) able to store results from

many types of experiments as relations between experimental or informational objects (clones, tissues, organisms, filters, images, etcetera). Relations are either based on the derivation of one object from one or more other objects, or on the results of experiments, linking different objects.

This gives a very flexible and expandable system that allows the storage of many different types of complex data. Data from the original RLDB, from the YAC screening centres, other external databases and experimental results have been converted and stored in a prototype of the new database.

We have started an experimental server that allows access to RLDB2 over the Internet (see Fig.). A client program can initiate a 'request to query' RLDB2 by connecting to the RLDB server, which then returns the RLDB2 'home page', a special document written in the Hyper Text Markup Language (HTML). The user can click on text or images (so called hyperlinks) that are included in this document in order to retrieve data from RLDB2 or other biological databases, to view RLDB files, to request items from the Reference Library System or

TABLE 3 Access to information from the RLDB

E-mail access

From the Reference Library DataBase (probe lists): GENOME@ICRF.ICNET.UK
From the EMBL file server (probe lists and IRX files): Netserv@EMBL-Heidelberg.DE

Anonymous ftp access

From the NCBI data repository at ncbi.nlm.nih.gov (130.14.20.1) in the directory/repository/RLDB (general info files) and/repository/RLDB/RLDBlist (probe lists) and/repository/RLDB/RLDBirx (IRX files)
From the EMBL ftp server at ftp.embl-heidelberg.de (192.54.41.33)

CD-ROM

On the NCBI contributed CD-ROM (contact NCBI for a copy)

Gopher access

BioGopher at EMBL (probe lists and IRX files). Link in your gopher server with following information:

Name=EMBNET BioInformation
Resource EMBL
Type=1
Port=70
Path=1/
Host=ftp.embl-heidelberg.de

Online access

Menu system of the UK HGMP Computer Resource Centre in Harrow (CRC registered users only): RLDB IRX database and probe data within IGD database
Telnet to menu.crc.ac.uk (192.68.153.50) or menu.crc.ac.uk 5888 (192.68.153.50 5888) from X-window platforms

WWW server

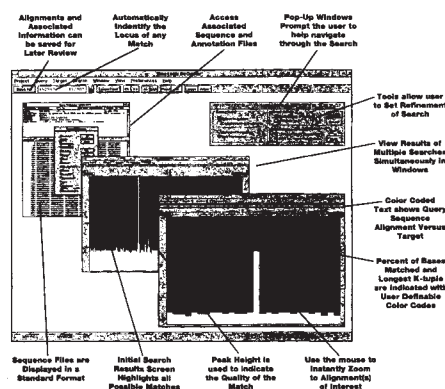
RLDB2 WWW server: point your WWW client to the URL: HTTP://gea.lif.icnet.uk/

to connect to other gophers and network servers.

If the RLDB2 is queried, the query is analysed, translated into standard query language (SQL) statements and the relevant data are retrieved from the database and converted into a new HTML document which is returned to the client process. Such HTML documents can contain normal text, hyperlinks to text or image files, to other objects in RLDB2 or to information in external databases (like the Genome Data Base (GDB), Online Mendelian Inheritance in Man (OMIM), GenBank, etcetera), which enables the user to retrieve additional information from the databases or to display a graphical image of the object. In case of physical or genetic maps or contigs, the displayed graphics can be used as a source for further queries by simply clicking on a map or contig member item in the image window.

The system allows non-registered users to view the public part of these data. Registered users can access the database with a password to access their own private or confidential data, to enter new

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DeCypher sequence similarity search system — see Time Logic.

to establish *a priori* search parameters. Results are presented utilizing a colour graphics interface so that significant matches may be rapidly seen on a single screen. DeCypher is said to perform sequence similarity searches with supercomputer speeds on a personal computer workstation platform. Time Logic says that accelerated search times are the result of the Sequence Similarity Engine technology that incorporates a proprietary parallel processing hardware architecture. Query sequences up to 128 kb in length can be cached on each board so that cosmid size sequences and clone sets can be compared to the entire data bank in just a few minutes. The system utilizes a modular design, which allows for current and future analysis requirements. DeCypher supports Windows 3.1.

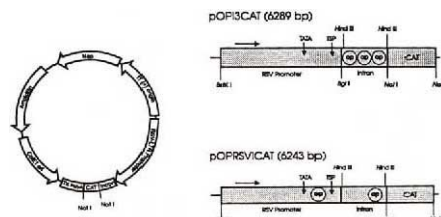
The Peptide Companion from CoshSoft/PeptiSearch (CSPS) is a new program for chemists working with peptides, peptidomimetics and proteins (Reader Service No. 105). It is said to be capable of handling polynucleotides, carbohydrates and any organic molecules composed of defined subunits. The program calculates molecular weights, results of elemental analysis (which can be corrected for various additional molecules presented in lyophilized products) with the option to analyse the analytical result for water and acetic acid content in the product. It evaluates amino acid composition and predicts mass spectral fragmentation of the given peptide or peptidomimetic. In addition, CSPS says that it predicts HPLC behaviour of the peptide (with eight different algorithms) and difficult sequences to synthesize. It also predicts conformational parameters and protein profiles (hydrophobicity, hydrophilicity, amphipathicity, ... nine different scales) of peptides and proteins of up to 3,000 amino acid residues (standard NIH format for sequence storage is used and therefore sequences from various databases can be imported). Mass spectra of peptidic products can be analysed to find the possible source of problems in their synthesis, and the composition of a peptidic and/or nonpeptidic library can be predicted. The program also serves as a database of hundreds of peptide (mostly biologically

active) structures and protecting groups. It has been programmed to 'understand' standard peptide language (for example, H-[Gly-Tyr(But)]5-Ile-Lys(CIZ)-Phe-CH₂NH-Ala-OH) and automatically translates three-letter code to one-letter code and *vice versa*.

Recognising that there is a need for faster, more up-to-date access to information by using the electronic highway called Internet, Trevigen, a new biotechnology research products company, is now making available the **gene regulation database** and related product information on the FTP server (Reader Service No. 106). Information on this anonymous FTP server, ftp.trevigen.com (164.109.10.23), is available free to all who have ftp access by logging in as "anonymous" and by using the Internet E-mail address as the password. Then go to the /pub/Tfactors directory. Those who do not have FTP access, but do have E-mail access can receive this information electronically by contacting the company. This information and the gene regulation database are also available in conventional formats.

■ Vectors

The LacSwitch inducible mammalian expression system developed by Stratagene is intended to provide a method for the inducible control of exogenous gene expression in



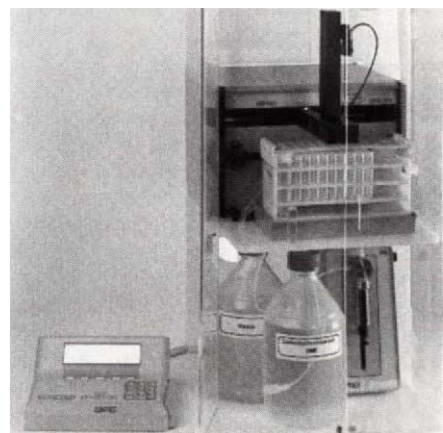
Stratagene's system for the inducible control of gene expression.

eukaryotic cells (Reader Service No. 107). It is comprised of a eukaryotic Lac-repressor-expressing vector (p3'SS) and two lac-operator-containing vectors (pOPRSVICAT and pOPISCAT). Several elements of the lactose operon have been modified to create a vector system that can tightly regulate, yet quickly induce expression. Both vectors contain a *Not I* cloning site, which allows insertion of a gene of interest, and the neomycin gene for G418 selection in cultured cells. After transfecting p3'SS and pOPRSVICAT or pOPISCAT into a cultured cell line, stable selection is maintained with hygromycin and geneticin. In the repressed state, the system is said to exhibit low basal expression of ~10–20 molecules per cell. Upon induction with the strong inducer, IPTG, expression of the operator-linked gene resumes within 4–8 h. The system's fast-acting, non-toxic inducer is said not to have adverse effects on eukaryotic cells, unlike methods that use heat shock, heavy metals or hormonal treatments.

Novagen has developed a series of **expression vectors** that encode a novel 15-amino acid leader useful for both detection and purification (Reader Service No. 108). These vectors make possible a nonradioactive homogenous rapid assay for recombinant protein in crude cell extracts. Inserts cloned into the S•Tag vectors are said to be expressed to a high level from a bacteriophage T7 promoter. The target protein is fused to a 15-amino acid S•Tag peptide, which can associate with exogenously added S-protein to produce fully functional RNase A. Novagen says that the RNase activity can be used to detect as little as 20 fmol of a fusion protein in a variety of formats. The pET-29a-c(+) vectors are designed for expressing S-tagged proteins in bacteria and the pCITE-3a-c(+) vectors permit optimal production of fusion protein *in vitro*. The S•Tag Rapid Assay Kit provides reagents for quantitative determination of fusion protein from a few microlitres of reticulocyte lysate or crude bacterial extract following a 5-min incubation. The S•Tag western blot kit is said to permit detection of subnanogram amounts of fusion protein on a western blot. Fusion proteins may be purified by chromatography or on an immobilized S•Protein matrix or the His•Bind resin.

■ Synthesis and sequencing

Laboratories needing just a small number of peptides may be interested in the new EPS 221 compact, **economy peptide synthesizer** from Abimed (Reader Service No. 109). This automated system is capable of unattended coupling of up to 45 amino acid derivatives, which are distributed on 1–3 flow-through columns. Abimed says that the synthesis procedure has been optimized for a 100-mmol scale regarding flow rates



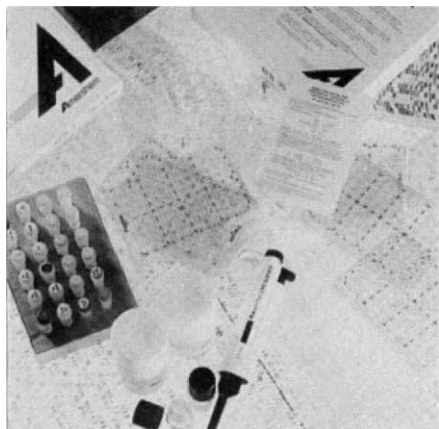
Abimed's automated peptide synthesizer.

and solvent consumption. An optimized chemistry protocol with a minimum number of variables is the default, although the protocol can be modified if needs be.

Hewlett-Packard's HP G1005A **protein sequencing system** is designed for the sequence analysis of low picomole-to-nanomole amounts of proteins and peptides

(Reader Service No. 110). The automated chemistry and analytical system comprises a protein sequencer, a sample preparation station and an online phenylthiohydantion amino-acid analyser based on the HP 1090M HPLC. A system controller, printer and consumables (sequencing reagents, solvents and columns) complete the system. The protein sequencer performs automated sequencing chemistry on a biphasic packed column sample support. The biphasic sequencer column is said to allow direct loading of samples from complex matrices or from dilute solutions, reducing the number of sample handling steps. Sample solutions may include many contaminants such as salts, amines, nonvolatile buffers, detergents and surfactants. Up to four sequencer columns can be used to perform back-to-back sequence runs without the need for intervention by the operator; reagents, solvents and gases may all be refilled while the sequencer is running.

To maximize the detection of DNA sequencing products, Amersham has developed the Hyperfilm range of high-resolution autoradiograph films (Reader Service No.



Autorad film for sequencing applications.

111). Unlike conventional X-ray film, Amersham says that Hyperfilm has a clear, colourless base that provides good contrast between exposed and unexposed areas and improves the information available from the gel. Hyperfilm-MP is recommended for routine sequencing with ^{35}S , ^{33}P and ^{32}P and is compatible with automatic film processors. It is available in rolls and sheets to suit all gel sizes. Hyperfilm-Bmax is said to be the film of choice for reading the maximum sequence with ^{35}S and ^{33}P . Only one side of the clear, colourless base is coated with an emulsion with a very high silver content. This is said to provide increased resolution and sensitivity over double-coated film. Again, a variety of sheet and roll sizes are available. Lastly, Hyperpaper- ^{35}S sequencing is a single-sided, paper-based material for use in ^{35}S and ^{33}P sequencing. Amersham says that it has the properties of film, which, coupled with its roll format and paper-like qualities, make it suitable for sequencing. Results are viewed

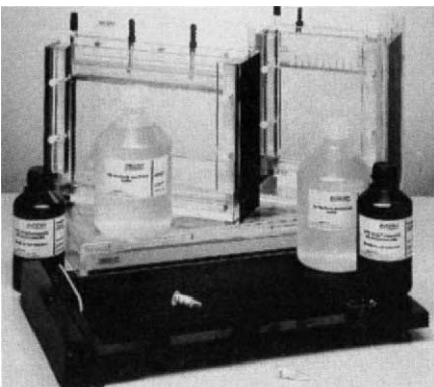
without a light box and can be written on, folded, photocopied and faxed.

Eurosequence is offering a new **custom service for fast internal sequence analysis** of (blocked) proteins (Reader Service No. 112). No HPLC or blotting is required. The company asks customers to provide a small piece of Coomassie Blue-stained SDS-PAGE gel containing the protein band. The service should prove especially useful for proteins that are otherwise difficult to isolate. Eurosequence says that internal sequences from many "difficult" proteins have been successfully determined in this way, even starting with 100 pmol of protein (although the company says that 250–500 pmol is a more suitable amount to ensure as many as possible unambiguous assignments in the final sequence analysis. Sequence information can be delivered within 2 weeks after receipt of the sample.

The SequiTherm Long-Read kit from Epicentre Technologies is a high-performance **sequencing kit** specifically designed for use with automated DNA sequencing machines (Reader Service No. 113). The kit features SequiTherm DNA polymerase, noted for producing more uniform band intensities and fewer dropouts than other thermostable polymerases. When SequiTherm is used in combination with the specially formulated Long-Read nucleotide mixes, the company says that runs of greater than 1,000 bases can be obtained. For example, the Long-Read kit has been used with the Li-Cor 4000L system to generate over 1,000 bases of sequence with a stated accuracy of greater than 99 per cent through base 800. Each kit contains enough reagents (except for labelled primers) for 100 sets of automated cycle-sequencing reactions.

■ Gel boxes

Ingeny's new **instrumentation for mutational scanning** by denaturing gradient gel electrophoresis (DGGE) and two-dimensional (2-D) DNA typing is designed to provide users with easy access to what can be quite complex methods (Reader Service No. 114). Up to 40 DGGE samples of two 2-D gels can be run simultaneously with this apparatus. A ceramic-coated electroblotting



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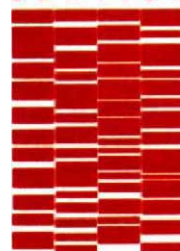
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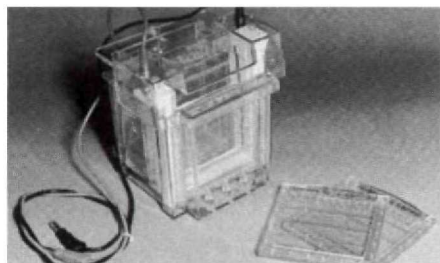
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Reader Service No.17

PRODUCT REVIEW

instrument allows direct 2-D analysis of single-copy genes for mutations. Image analysis software run under Windows is intended to facilitate the interpretation of complex gel or hybridization patterns.

Novex has recently modified its original X-Cell mini-cell electrophoresis unit, which can be obtained through British Biotechnology, to include a variety of safety,



Excel with the X-Cell II mini-cell unit.

durability and performance features (*Reader Service No. 115*). The new X-Cell II unit is now said to be shatterproof and has newly designed power cords and gaskets. These features make it impossible to touch any part of the unit and complete a live circuit, BBt says. An interlocking assembly breaks all possible electric circuits when the lid is removed. Finally, buffer chambers have been altered to facilitate buffer and sample loading and also to improve heat dissipation.

A new 8-page brochure from Perkin-Elmer describes the company's Model 270A-

HT high-throughput capillary electrophoresis system (*Reader Service No. 116*). The fully automated system can be used to complement HPLC or for implementing a quantitative method that facilitates and automates slab-gel electrophoresis protocols. At the heart of the system is the thermostatted, programmable autosampler, which is designed to provide precise temperature control, flexible heating, cooling and evaporation control for both samples and buffers, and high speed and throughput. Information is also offered on preparative sample collection of high-resolution separations and the ability to re-analyse automatically fractions. The Model 270A-HT also features a sensitive, low noise benchmark HPLC detector design and software that provides a choice of data systems, built-in error logging and a manual mode.

■ Thermal cyclers

Welltemp, the new thermal cycler from Denley Instruments, features a heated lid to prevent evaporation and remove the need for oil overlays (*Reader Service No. 117*). Up to 99 programmes, each with 99 segments for determining time, temperature and ramping rates, can be defined and stored. Welltemp includes interchangeable blocks capable of accommodating up to 40 0.5-ml microcentrifuge tubes and 96 0.25-ml polypropylene microreaction tubes. In addition, different types of microplate (including the Falcon round-bottom plate) and slides can also be used. The peltier unit is said to

provide temperature control that is accurate to $\pm 0.1^\circ\text{C}$, with a uniformity of temperature across the block of better than 1°C extending to the peripheral zones. Ramping can be controlled from 1°C per second to 1°C per minute during both heating and cooling.

The small, compact Mastercycler 5330 from Eppendorf-Netheler-Hinz is a thermostat specially designed for temperature cycling that features two blocks each with separate control (*Reader Service No. 118*). Heating and cooling is by peltier technology and is independent of external cooling. The device accommodates two lots of 27 samples in 0.5 ml Safe-Lock microcentrifuge tubes. The company says that no mineral oil is required between the tube and block due to the snug fit of the Safe-Lock tubes in the block. A temperature adjustment rate of greater than 2°C per second is said to permit run times of about an hour per experiment.

■ Catalogues

The 7th edition of the ATCC/NIH repository catalogue of human and mouse DNA probes and libraries is now available from the American Type Culture Collection (*Reader Service No. 119*). This latest edition lists the following: probes and cloned



ATCC's probes and libraries.

genes for human or murine loci, oligonucleotides, human chromosome-specific genomic libraries, cDNA and genomic libraries, oncogene/transforming protein probes and clones, selected probes detecting polymorphism, bacterial hosts for transformation or plating libraries, human cDNA clones, and the CEPH/ATCC chromosome 1 mapping kit. Literature citations and general information on the handling of cultures is provided. Expanded information on the materials listed in the catalogue is available via modem, Internet and diskette.

Advanced ChemTech's new product catalogue features amino acids, resins and reagents for peptide synthesis, including Boc- and Fmoc-amino acids, D-amino acids, unusual amino acids, amino acid alcohols and N-methyl-amino acids (*Reader Service No. 120*). The catalogue lists a wide range of Boc- and Fmoc-linked resins and functionalized resins for SPPS, including the MAP (multiple antigenic peptide) system in Wang and PAM resin supports; Knorr, Oxime and PAL resins; and Rink, MBHA, BHA, Merrifield and aminomethyl resins. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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